CONDITIONS FOR ACTIVATION OF GROUP I METABOTROPIC GLUTAMATE RECEPTORS IN RAT HIPPOCAMPUS

DISSERTATION

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The unity of knowledge and action

Wang Yangming

Dedicated to my parents

Zusammenfassung

Metabotrope Glutamatrezeptoren (mGluRs) gehören zur Familie der G-Protein-gekoppelten Rezeptoren und spielen eine wichtige Rolle im Rahmen synaptischer Plastizität, Entwicklung des Nervensystems und Neurodegeneration. mGluRs der Klasse 1 (mGluR1 und mGluR5) können selektiv durch 3,5-Dihydroxyphenylglycin (3,5-DHPG) aktiviert werden. Klasse 1 mGluRs können im Gehirn sowohl prä- als auch postsynaptisch lokalisiert sein; am häufigsten kommen sie in der postsynaptischen Dichte asymmetrischer, glutamaterger Synapsen vor.

Ziel der vorliegenden Arbeit ist herauszufinden, unter welchen Bedingungen Klasse 1 mGluRs im Hippocampus der Ratte aktiviert werden.

In Kapitel 2 zeige ich, dass Klasse 1 mGluRs in CA1 Pyramidenzellen hippocampaler Schnittkulturen nicht tonisch durch basale Glutamatlevel aktiviert werden.

In Kapitel 3 lege ich dar, dass die Aktivierung einer einzelnen CA3 Pyramidenzelle kein mGluR-LTP im direkt mit ihr verbundenen CA1 Pyramidalneuron induziert. Anhand von Paarableitungen mit extrazellulärer Stimulation lässt sich schliessen, dass schätzungsweise 8 aktive CA3-Eingänge auf eine CA1 Pyramidenzelle konvergieren müssen um in dieser mGluR-LTP auszulösen.

In Kapitel 4 zeige ich, dass die Aktivierung einer einzelnen CA3 Pyramidenzelle keinen durch Klasse 1 mGluRs vermittelten somatischen Strom in einem synaptisch verbundenen Interneuron hervorruft. Klasse 1 mGluRs in Interneuronen des Hippocampus werden durch Theta-Oszillationen aktiviert und modulieren deren Frequenz.

Summary

Metabotropic glutamate receptors (mGluRs) are a family of G-protein-coupled receptors and have been shown to play an important role in synaptic plasticity, neuronal development, and neurodegeneration. Group I mGluRs (mGluR1 and mGluR5) are selectively activated by 3,5-dihydroxyphenylglycine (3,5-DHPG). Although Group I mGluRs can be located at both presynaptic and postsynaptic sites in the brain, they are mostly observed at the periphery of the postsynaptic densities of asymmetrical glutamatergic synapses

The aim of my thesis project is to determine the conditions required for activation of group I mGluRs in rat hippocampus.

In chapter 2, I show that group I mGluRs in CA1 pyramidal cells are not tonically activated by ambient glutamate in hippocampal slice cultures.

In chapter 3, I show that activation of a single CA3 pyramidal cell does not induce mGluR-LTD in a connected CA1 pyramidal cell. An estimate based on data obtained from paired recording and extracellular stimulation indicates that 8 active CA3 inputs are required to induce mGluR-LTD in a targeted CA1 pyramidal cell.

In chapter 4, I show that activation of a single CA3 pyramidal cell does not evoke a somatic current mediated by group I mGluRs in a synaptically connected interneuron. I examine the roles of group I mGluRs in hippocampal interneurons during network oscillations and find that their activation modulates theta frequency.

Table of contents

Chapter 1

Introduction

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) and it acts through a variety of ionotropic (ligand-gated cation channels) and metabotropic (G-protein coupled) receptors (Fig. 1.1, adapted from Kew and Kemp, 2005).

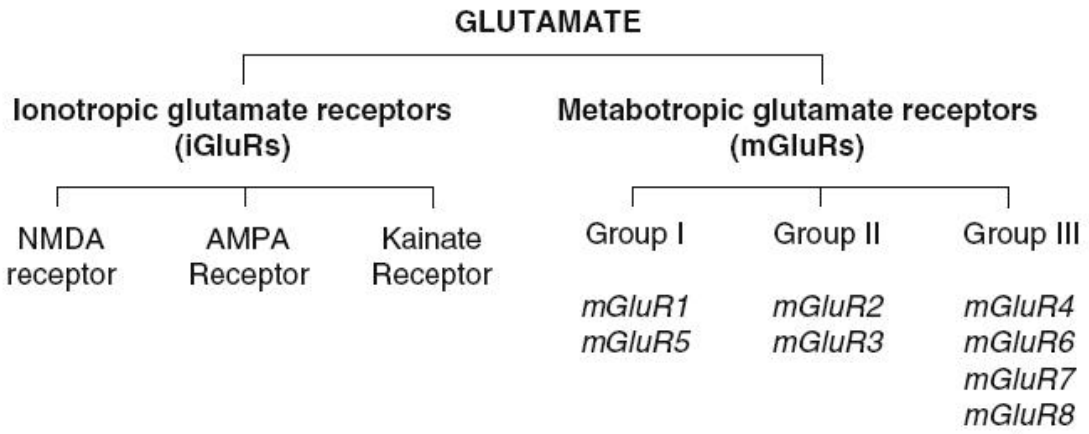

Figure 1.1 Glutamate receptors. Adapted from Kew and Kemp, 2005.

1.1 Ionotropic glutamate receptors

Ionotropic glutamate receptors (iGluRs) are ion channels permeable to cations and are subdivided into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors based on agonist preference (Bettler and Mulle, 1995; Dingledine 1999; Kew and Kemp, 2005; Mayer, 2005; Köhr, 2006; Pinheiro and Mulle, 2006; Sprengel, 2006).

iGluRs are tetramers with each subunit possessing an extracellular amino terminal domain, followed by a first transmembrane domain and then a pore forming membrane-residing domain that does not cross the membrane but forms a re-entrant loop entering from and exiting to the cytoplasm. The second and third transmembrane domains are linked by a large extracelluar loop and the

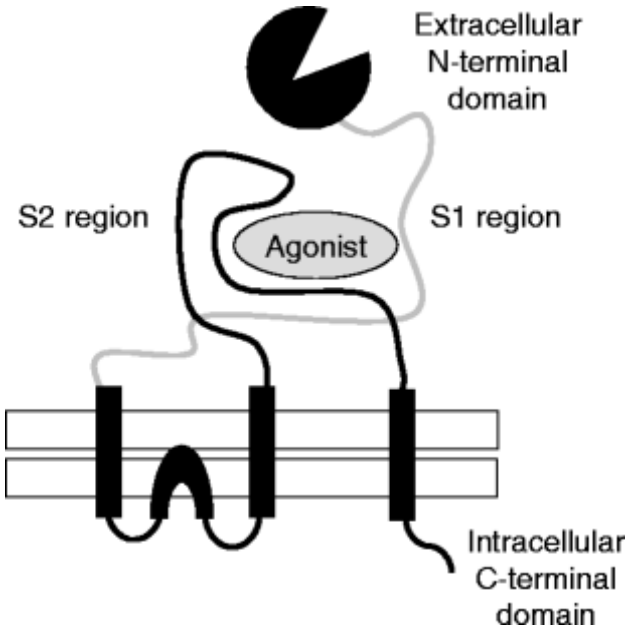

Figure 1.2 Schematic drawing of an Ionotropic glutamate receptor subunit. Taken from Kew and Kemp, 2005.

third transmembrane domain is followed by an intracellular carboxy-terminus. The structure of the iGluR ligand binding domain is located in a pocket formed between the extracellular amino terminus (S1 domain) and the extracellular loop between transmembrane domains 3 and 4 (S2 domain) (Fig. 1.2) (Dingledine et al. 1999; Mayer and Armstrong 2004; Kew and Kemp, 2005).

1.1.1 NMDA receptors

NMDA receptors (NMDARs) play pivotal roles in synaptic plasticity, synapse formation and neuronal cell death. A unique property of the NMDAR is its voltage-dependent activation, a result of ion channel block by extracellular Mg^{2+} ions at physiological concentrations. NMDARs require the co-agonist glycine or D-serine. Activation of the NMDAR results in the opening of an ion channel that is nonselective to cations, which allows voltage-dependent flow of Na^{+} and small

amounts of Ca^{2+} ions into the cell and K^{+} out of the cell. Seven NMDAR subunits have been identified: NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B. There are eight different NR1 subunits generated by alternative splicing from a single gene. All NMDARs are heteromeric assemblies composed of two NR1 subunits in combination with at least one type of NR2. The NR3 subunit does not form functional receptors alone, but can co-assemble with NR1/NR2 complexes. NMDAR functional properties are determined by subunit composition (Cotman and Monaghan, 1988; Daw et al., 1993; Mori and Mishina, 1995; Dingledine 1999; Cull-Candy et al., 2001; Debanne et al., 2003; Riedel et al., 2003; Wenthold et al., 2003; Mayer, 2005; Groc and Choquet, 2006; Köhr, 2006; Lau and Zukin, 2007; Paoletti and Neyton, 2007).

1.1.2 AMPA receptors

AMPA receptors (AMPARs) are the principal channels that mediate fast excitatory synaptic transmission. AMPARs are composed of four types of subunits, which combine to form tetramers. These subunits are designated as GluR1, GluR2, GluR3 and GluR4, alternatively called GluRA, GluRB, GluRC and GluRD. Most AMPA receptors contain the GluR2 subunit. The AMPAR is permeable to calcium and other cations, such as sodium and potassium. The AMPAR's permeability to calcium is governed by the GluR2 subunit. Functional AMPA receptors are located predominantly in the postsynapse but are also found at extrasynaptic sites and occasionally in the presynapse. The glutamate-mediated transmission efficiency of synaptic AMPARs is influenced by their subunit composition, post-transcriptional and post-translational modifications, the number of synaptic AMPARs, and auxiliary proteins. AMPAR function is regulated over long periods (days to weeks) in response to chronic changes in the level of neuronal activity, and over short periods (seconds to minutes) in response to acute changes in synaptic activity, as seen during long-term potentiation (LTP) or long-term depression (LTD). Modulation of AMPAR function occurs through two distinct mechanisms: modulation of ion-channel properties and regulation of

synaptic targeting of the receptors. Both processes are regulated by the phosphorylation of AMPARs (Bettler and Mulle, 1995; Dingledine 1999; Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003; Debanne et al., 2003; Esteban, 2003; Malenka, 2003; Riedel et al., 2003; Schenk and Matteoli, 2004; Mayer, 2005; Wang et al., 2005; Groc and Choquet, 2006; Nicoll et al., 2006; Sprengel, 2006; Derkach et al., 2007; Greger et al., 2007; Greger and Esteban, 2007; Kessels and Malinow, 2009; Santos et al., 2009).

1.1.3 Kainate receptors

Kainate receptors (KARs) play important roles in regulating synaptic transmission and neuronal excitability. KARs are localized in postsynapse and presynapse. Postsynaptic KARs mediate synaptic currents of small amplitude and slow rise and decay kinetics compared with AMPAR-mediated currents. The ion channel formed by kainate receptors is permeable to sodium and potassium ions. Presynaptic KARs can modulate neurotransmitter release such as GABA and glutamate. Therefore presynaptic KARs can facilitate presynaptic forms of short and long term synaptic plasticity. KARs are tetrameric receptors composed of various combinations of five subunits GluR5, GluR6, GluR7, KA1 and KA2. GluR5, GluR6 and GluR7 can form functional homomeric and heteromeric receptor channels and have multiple isoforms derived from alternative splicing and RNA editing. GluR5 has two N-terminal splice variants (GluR5-1 and GluR5-2) and three C-terminal splice variants in the cytoplasmic domain (GluR5a, GluR5b and GluR5c). GluR6 has two C-terminal splice variants (GluR6a and GluR6b). GluR7 has two C-terminal splice variants (GluR7a and GluR7b). KA1 and KA2 can only form functional receptors by combining with one of the GluR5-7 subunits. No splice variants for KA1 and KA2 have been found (Bettler and Mulle, 1995; Bleakman, 1999; Chittajallu et al., 1999; Dingledine 1999; Kullmann, 2001; Kamiya, 2002; Debanne et al., 2003; Huettner, 2003; Lerma, 2003, 2006; Riedel et al., 2003; Isaac et al., 2004; Jaskolski, 2005; Mayer, 2005; Pinheiro and Mulle, 2006, 2008; Coussen, 2009).

1.2 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are a family of G-protein-coupled receptors and have been shown to play an important role in synaptic plasticity, learning and memory, neuronal development and neurodegeneration (Nakanishi, 1994; Pin and Bockaert, 1995; Pin and Duvoisin, 1995; Conn and Pin, 1997; Anwyl, 1999; Bordi and Ugolini, 1999; De Blasi et al., 2001; Hermans and Challiss, 2001; Dale et al., 2002; Gerber, 2002; Valenti et al., 2002; Conn, 2003; Conn et al., 2005; Kew and Kemp, 2005; Dhami and Ferguson, 2006; Ferraguti and Shigemoto, 2006; Gerber et al., 2007).

1.2.1 Classification

Eight distinct mammalian mGluRs have been reported and have been divided into three subgroups based on sequence similarities, pharmacological agonists and antagonists, and signal transduction pathways (Nakanishi, 1994; Pin & Bockaert, 1995; Pin & Duvosin, 1995; Conn & Pin, 1997; Dale et al., 2002). They are Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7, and mGluR8) (Fig. 1.3). Splice variants have been reported for mGluR1, mGluR3, mGluR5, mGluR6, mGluR7 and mGluR8 (Fig. 1.3; Pin and Duvoisin 1995; Kew and Kemp, 2005; Ferraguti and Shigemoto, 2006).

Group I mGluRs, which include mGluR1 and mGluR5, are selectively activated by 3,5-dihydroxyphenylglycine (3,5-DHPG). Via G-protein-dependent pathways, group I mGluRs are positively coupled to the phospholipase C (PLC), inositol-1,4,5-trisphosphate (IP_3) formation, activation of protein kinase C (PKC) and release of calcium from intracellular stores (Abdul-Ghani et al., 1996; Conn and Pin, 1997; Bordi and Ugolini, 1999; Hermans and Challiss, 2001; Dhami and Ferguson, 2006). Group I mGluRs can also activate intracellular signals independent of G proteins, via tyrosine kinase Src (Heuss et al., 1999; Benquet

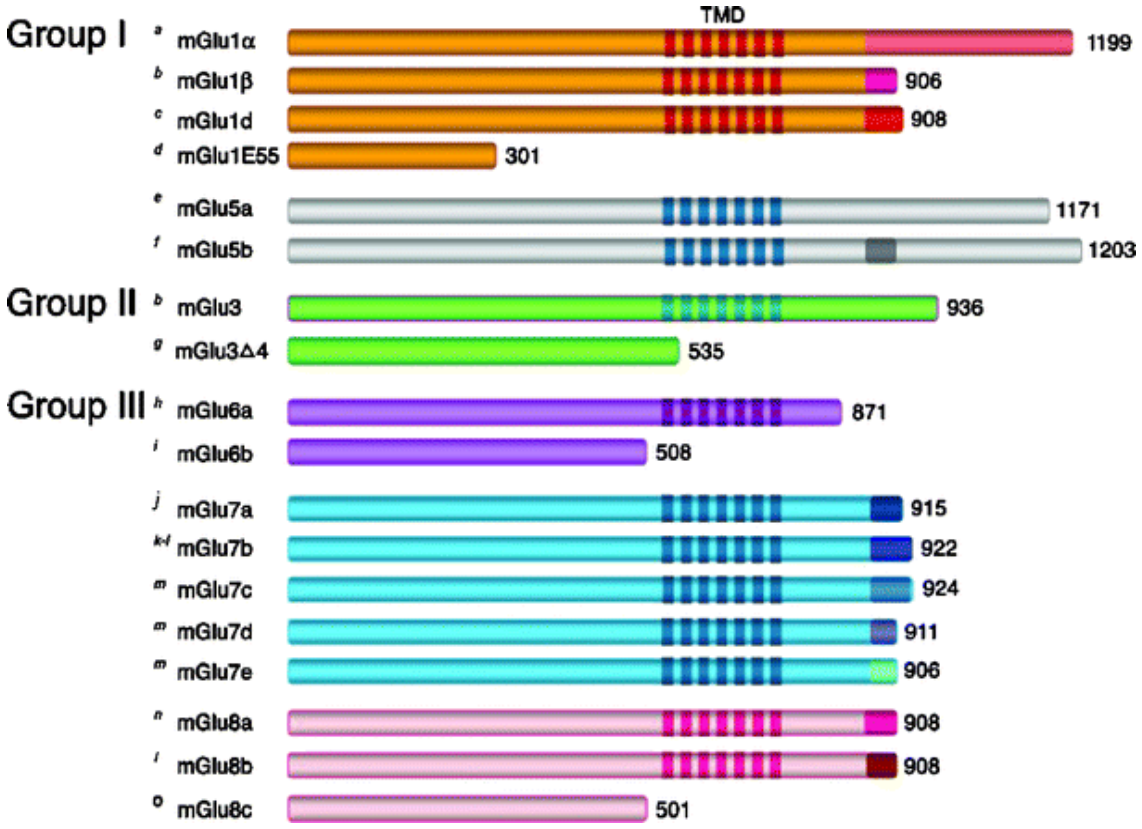

Figure 1.3 Schematic drawing of alternative splice variants of metabotropic glutamate receptors. The different colors in the C-terminus indicate the changes in amino acid sequence between isoforms attributable to alternative splicing. TMD, transmembrane domain. Taken from Ferraguti and Shigemoto, 2006.

et al., 2002; Gerber, 2002; Krause et al., 2002; Gee et al., 2003; Gee and Lacaille, 2004; Gerber et al., 2007).

Group II mGluRs, which include mGluR2 and mGluR3, are selectively activated by DCG-IV or LY379268 and are negatively coupled to adenylyl cyclase. Group II mGluRs negatively modulate glutamate and GABA neurotransmission (Anwyl, 1999; Schoepp, 2001; Kew and Kemp, 2005; Dhami and Ferguson, 2006; Ferraguti and Shigemoto, 2006).

Group III mGluRs, which include mGluR4, mGluR6, mGluR7, and mGluR8, are

selectively activated by L-(+)-2-amino-4-phosphonobutyrate (L-AP4) and are negatively coupled to adenylyl cyclase. Group III mGluRs negatively modulate glutamate and GABA neurotransmission (Anwyl, 1999; Schoepp, 2001; Kew and Kemp, 2005; Dhami and Ferguson, 2006; Ferraguti and Shigemoto, 2006).

1.2.2 Structure

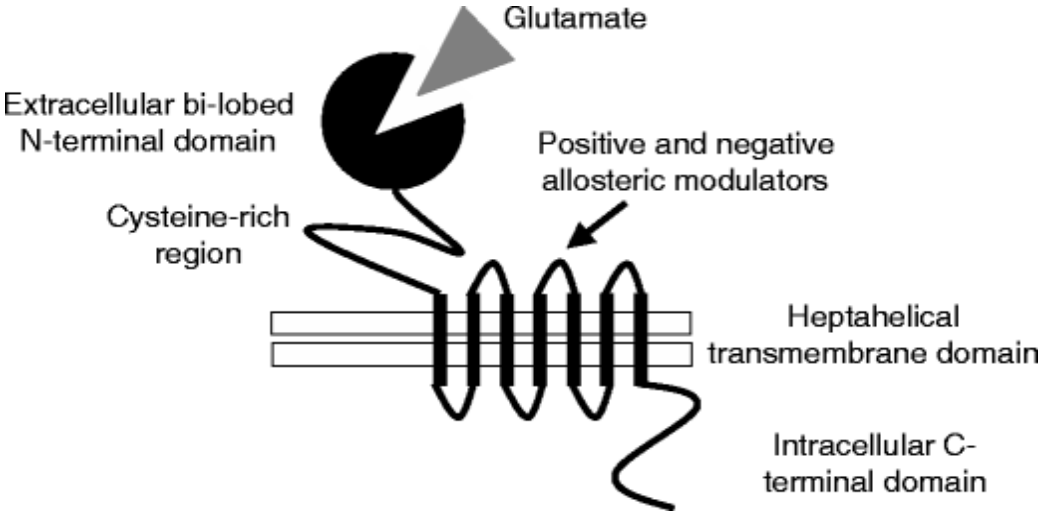

Figure 1.4 Schematic drawing of the structure of an mGluR. Taken from Kew and Kemp, 2005.

The topology of mGluRs is shown in Fig. 1.4, which includes an N-terminal extracellular domain, three extracellular and three intracellular loops and a cytoplasmic C-terminal tail, separated by seven transmembrane domains. The large N-terminal extracellular domain contains the glutamate-binding site. The transmembrane heptahelical domain contains the binding sites for positive and negative allosteric modulators. The intracellular C-terminal domain interacts with cytoskeletal, scaffolding and signaling proteins (e.g., G-proteins) and with integral membrane receptors. These proteins are responsible for the correct targeting of mGluRs to specific sub-cellular compartments, coupling to effector proteins,

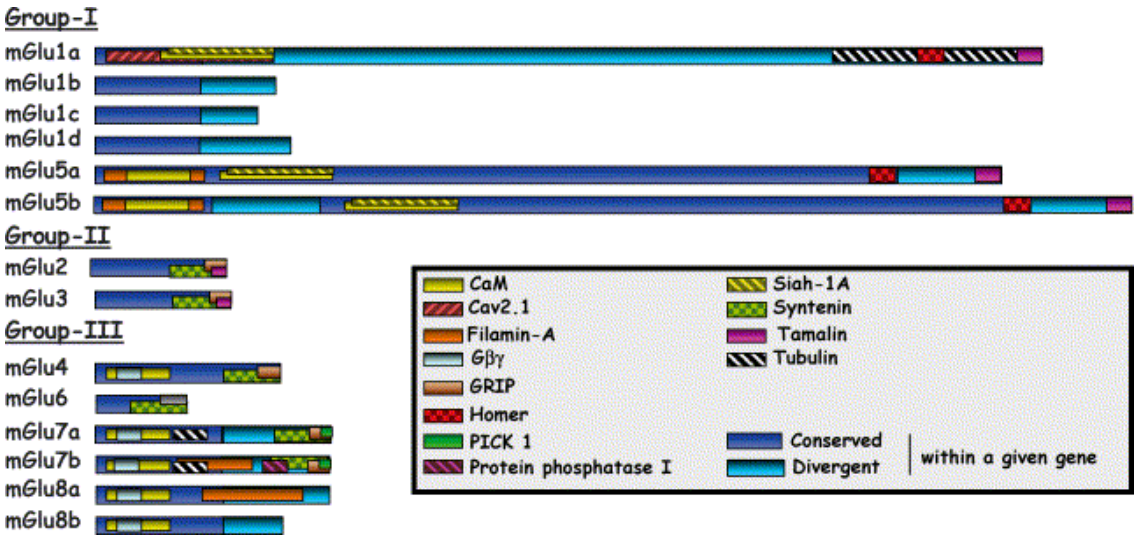

Figure 1.5 Binding domains of mGluRs-interacting proteins in the C-terminus of mGluRs. *Abbreviations*: CaM, Calmodulin; Siah-1A, seven in absentia homolog-1A; GRIP, glutamate receptor interacting protein; PICK1, protein interacting with C kinase 1. Taken from Fagni et al. 2004.

modulation of intracellular signalling and the recycling of mGluRs (Fig. 1.5) (De Blasi et al., 2001; De Blasi et al., 2001; Hermans and Challiss, 2001; Jingami et al., 2003; Fagni et al. 2004; Kew and Kemp, 2005; Ferraguti and Shigemoto, 2006).

1.2.3 Function

mGluRs have been implicated in diverse functions in the mammalian CNS (Pin and Duvoisin, 1995; Conn and Pin, 1997; Anwyl, 1999; Conn, 2003; Kew and Kemp, 2005; Dhami and Ferguson, 2006; Ferraguti and Shigemoto, 2006). mGluRs mediate slow excitatory (Charpak and Gähwiler, 1991; Glaum and Miller 1992; McCormick and von Krosigk 1992; Eaton et al. 1993; Congar et al., 1997; Gee et al., 2003; Wu et al. 2004) and inhibitory (Fiorillo and Williams 1998) responses. mGluRs regulate calcium channels (Swartz and Bean 1992; Sahara and Westbrook 1993; Chavis et al. 1994), potassium channels (Charpak et al. 1990; Guérineau et al., 1994; Shirasaki et al. 1994; Gereau and Conn 1995b;

Netzeband et al. 1997) and non-selective cation channels (Guérineau et al. 1995; Congar et al. 1997). mGluRs regulate the trafficking of ionotropic glutamate receptors (Lan et al. 2001; Snyder et al. 2001) and modify NMDAR-mediated synaptic transmission (Awad et al. 2000; Aniksztejn et al., 1991, 1992; Bleakman et al., 1992; Harvey and Collingridge, 1993; Pisani et al., 1997, 2001; Ugolini et al., 1997, 1999; Yu et al., 1997; Contractor et al., 1998; Wang et al., 1998; Zhong et al., 2000; Benquet et al., 2002; Zho et al., 2002; Grishin et al., 2004; Guo et al. 2004; Harney et al. 2006; Ireland and Abraham, 2009). mGluRs inhibit (Baskys and Malenka 1991; Desai and Conn 1991; Schrader and Tasker 1997; Wittmann et al. 2001; Xi et al. 2003; Acuna-Goycolea et al. 2004; Chu and Moenter 2005) and facilitate transmitter release (Rodriguez-Moreno et al. 1998; Morsette et al. 2001; Herrero et al. 1992). Activation of mGluRs can induce LTP (Bortolotto and Collingridge 1993; O'Connor et al. 1995; Manahan-Vaughan 1997; Raymond et al. 2000; Chiamulera et al., 2001; Miura et al. 2002; Gubellini et al., 2003; Sourdet et al., 2003; Topolnik et al., 2006; Anwyl, 2009) and LTD (Linden et al. 1991; Stanton et al., 1991; Bashir et al., 1993b; Kato 1993; Bolshakov and Siegelbaum 1994; Conquet et al. 1994; Shigemoto et al. 1994; Kemp and Bashir, 1997, 1999; Oliet et al., 1997; Palmer et al., 1997; Nicoll et al., 1998; Fitzjohn et al., 1999; Huber et al., 2000, 2001; Lin et al. 2000; Gubellini et al., 2001; Xiao et al., 2001; Daw et al., 2002; Feinmark et al., 2003; Otani et al. 2002; Gallagher et al., 2004; Moult et al., 2006; Grueter et al., 2007; Cho et al., 2008; Jo et al., 2008; Neyman and Manahan-Vaughan, 2008; Wang et al., 2008; Waung et al., 2008; Holbro et al., 2009). mGluRs play an important role in memory formation (Aiba et al. 1994; Packard et al. 2001) and the regulation of neuronal development (Pin and Duvoisin 1995; Hensch and Stryker 1996; Conn and Pin 1997; Kano et al. 1997; Plenz and Kitai 1998; Catania et al. 2001; Hannan et al. 2001). mGluRs are involved in various diseases such as anxiety and stress disorders (Linden et al. 2002; Swanson et al. 2005), epilepsy (Sansig et al. 2001), excitotoxic neuronal death (Bruno et al. 2000), hypoxic brain damage (Poli et al. 2003) and fragile X mental retardation (Bear et al. 2004).

1.3 Group I metabotropic glutamate receptors

1.3.1 Distribution of group I mGluRs in the central nervous system

mGluR1 has been found in hippocampal neurons, in Purkinje cells of the cerebellar cortex, in mitral/tufted cells of the olfactory bulb, as well as in the lateral septum, globus pallidus, entopeduncular nucleus, ventral pallidum, magnocellular preoptic nucleus and thalamus (Martin et al. 1992; Shigemoto et al. 1992; Hubert et al. 2001).

Differential mRNA expression of mGluR1 splice variants has been observed (Berthele et al. 1998, 1999). Strong expression of the mGlu1α splice variant has been found in Purkinje cells, hippocampal interneurons, mitral and tufted cells, thalamic neurons and in the substantia nigra (Martin et al. 1992; Baude et al. 1993; Fotuhi et al. 1993; Ferraguti et al. 2004). The mGlu1β splice variant is intensively expressed in CA3 hippocampal pyramidal neurons, granule cells of the dentate gyrus and lateral hypothalamus (Ferraguti et al. 1998; Mateos et al. 1998).

Protein levels of mGluR1α are found to increase progressively during pre- and post-natal development (Catania et al. 1994; van den Pol et al. 1994; Lopez-Bendito et al. 2002; Shigemoto et al. 1992).

In contrast, the expression of mGluR5 is low in the cerebellum (Neki et al. 1996; Negyessy et al. 1997). High expression has been found in the cerebral cortex, hippocampus, subiculum, olfactory bulb, anterior olfactory nucleus, olfactory tubercle, striatum, nucleus accumbens and lateral septal nucleus (Abe et al. 1992; Shigemoto et al. 1993; Romano et al. 1995; Bordi and Ugolini, 1999). In the hippocampus, mGluR5 is mainly expressed in interneurons as well as in dendritic fields of pyramidal and granule cells (Kerner et al. 1997; Shigemoto et al. 1997).

mGluR5 increases perinatally, with a peak around the second postnatal week, and decreases thereafter (Catania et al. 1994; Romano et al. 1996; Lopez-Bendito et al. 2002). Changes in mGluR5 expression have been associated with a gradual decrease in mGluR5a and an increase in mGluR5b. mGlu5Ra is found most abundantly in the young rat, whereas mGlu5Rb predominates in the adult rat (Joly et al. 1995; Minakami et al. 1995; Romano et al. 1996).

1.3.2 Subcellular localization of group I mGluRs

At the electron-microscopic level, although Group I mGluRs can be located at both presynaptic and postsynaptic sites in the brain (Gereau and Conn,1995a; Manzoni and Bockaert, 1995; Romano et al. 1995; Jia et al. 1999; Simkus and Stricker, 2002; White et al., 2003; Park et al., 2004; Musante et al., 2008), they are mostly observed at the periphery of the postsynaptic densities of asymmetrical glutamatergic synapses (Baude et al., 1993; Shigemoto et al., 1993, 1997; Nusser et al. 1994; Lujan et al., 1996, 1997; Vidnyánszky et al. 1996; Liu et al. 1998).

mGluR1 has been detected mostly postsynaptically in neurons in the hippocampus (Baude et al. 1993; Lujan et al. 1996, 1997; Hanson and Smith 1999), cerebral cortex (Ong et al. 1998), striatum (Hanson and Smith 1999), thalamus (Martin et al. 1992; Godwin et al. 1996; Liu et al. 1998), hypothalamus (van den Pol et al. 1994), cerebellar cortex (Martin et al. 1992; Baude et al. 1993; Gorcs et al. 1993; Nusser et al. 1994; Lujan et al. 1996 1997; Jaarsma et al. 1998; Mateos et al. 2000) and dorsal cochlear nucleus (Petralia et al. 1996; Jaarsma et al. 1998). At the head of Purkinje cell spines in the rat cerebellar cortex, about half of the immunogold particles for mGluR1α are localized perisynaptically, i.e. within a 60-nm annulus surrounding the edge of synapses, whereas the remaining particles are localized extrasynaptically at more distant positions (Lujan et al. 1997).

mGluR5 has been detected mainly in somatic and dendritic domains in the hippocampus (Lujan et al. 1996, 1997; Hanson and Smith 1999), basal ganglia (Shigemoto et al. 1993; Hanson and Smith 1999), thalamus (Godwin et al. 1996; Liu et al. 1998), hypothalamus (Romano et al. 1995; van den Pol et al. 1995), cerebellar cortex (Négyessy et al. 1997), dorsal cochlear nucleus (Petralia et al. 1996) and dorsal horn of the spinal cord (Vidnyánszky et al. 1994; Jia et al. 1999). In a few studies, mGluR5 immunoreactivity has been reported not only in somatodendritic domains of neurons, but also in axons (Romano et al. 1995) or vesicle-containing profiles (Jia et al. 1999). At the head of CA1 pyramidal neuron spines, about one fourth of the immunogold particles for mGluR5 are observed at the periphery of the postsynaptic density (PSD) of asymmetrical synapses (Lujan et al. 1996, 1997).

In the developing nervous system, mGluR5 is preferentially found in unmyelinated axons and pyramidal cell dendrites in the first two postnatal weeks. As development proceeds, the number of mGluR5-immunolabelled unmyelinated axons is significantly reduced and the mGluR5 becomes more widely distributed on dendritic spines (Liu et al. 1998; Lopez-Bendito et al. 2002; Hubert and Smith 2004).

1.3.3 Function of group I mGluRs

Group I mGluRs have been shown to play an important role in neuronal development (Catania et al., 1991; Plenz & Kitai, 1998; Flint et al., 1999; Hannan et al., 2001), synaptic plasticity (Zhong et al., 2000; Wu et al., 2001; Gubellini et al., 2003), as well as in several neurological and neurodegenerative disorders (Kingston et al., 1999; Bruno et al., 2001), such as chronic pain (Bhave et al., 2001; Karim et al., 2001; Zhou et al., 2001), epilepsy (Chapman et al., 2000), multiple sclerosis (Geurts et al., 2003), amyotropic lateral sclerosis (Laslo et al., 2001), Huntington's disease (Calabresi et al., 1999) and Parkinson's disease (Awad et al., 2000).

The activation of group I mGluRs has also been shown to play an important role in long- and short-term synaptic plasticity. Two forms of long-term synaptic plasticity, LTP and LTD, are observed in several brain regions and have been proposed to represent the cellular mechanisms for learning and behaviour. The activation of group I mGluRs is required for the induction of LTP (Bashir et al., 1993a; Aiba et al., 1994; Conquet et al., 1994; O'Connor et al., 1995; Lu et al., 1997; Raymond et al., 2000; Chiamulera et al., 2001; Gubellini et al., 2003; Sourdet et al., 2003; Topolnik et al., 2006; Anwyl, 2009) and LTD (Linden et al. 1991; Bashir et al., 1993b; Kato 1993; Bolshakov and Siegelbaum 1994; Conquet et al., 1994; Shigemoto et al. 1994; Kemp and Bashir, 1997, 1999; Oliet et al., 1997; Palmer et al., 1997; Nicoll et al., 1998; Fitzjohn et al., 1999; Huber et al., 2000, 2001; Gubellini et al., 2001; Xiao et al., 2001; Daw et al., 2002; Feinmark et al., 2003; Gallagher et al., 2004; Moult et al., 2006; Grueter et al., 2007; Cho et al., 2008; Jo et al., 2008; Neyman and Manahan-Vaughan, 2008; Wang et al., 2008; Waung et al., 2008; Holbro et al., 2009). In the hippocampus, mGluR1-deficient mice exhibit either normal or only slightly reduced CA1 and dentate LTP, but impaired CA3 mossy fiber LTP (Aiba et al., 1994; Conquet et al., 1994). Conversely, mGluR5-deficient mice display a reduction in CA1 hippocampal LTP, but demonstrate normal CA3 mossy fiber LTP and have a reduced performance in spatial learning tasks (Lu et al., 1997).

It was reported that group I mGluRs modulate NMDA receptor responses in CNS (Awad et al. 2000; Aniksztejn et al., 1991, 1992; Bleakman et al., 1992; Harvey and Collingridge, 1993; Pisani et al., 1997, 2001; Ugolini et al., 1997, 1999; Yu et al., 1997; Contractor et al., 1998; Wang et al., 1998; Zhong et al., 2000; Lan et al., 2001; Snyder et al., 2001; Benquet et al., 2002; Zho et al., 2002; Grishin et al., 2004; Guo et al., 2004; Harney et al., 2006; Ireland and Abraham, 2009), which may represent an additional mechanism through which NMDA-dependent synaptic plasticity can be modulated.

1.4 Long-term potentiation and long-term depression

1.4.1 Long-term potentiation

Long-term potentiation (LTP) of the chemical synaptic transmission is the enhancement of signal transmission between two nerve cells which lasts for an extended period of time (minutes to hours *in vitro* and hours to days and months *in vivo*). LTP is widely considered as the major cellular basis of learning and memory (Teyler and DiScenna, 1987; Bliss and Collingridge, 1993; McNaughton, 1993; Martinez and Derrick, 1996; Redman, 1996; Malenka and Nicoll, 1999; Debanne et al., 2003; Malenka and Bear, 2004; Cooke and Bliss, 2006; Kullmann and Lamsa, 2007).

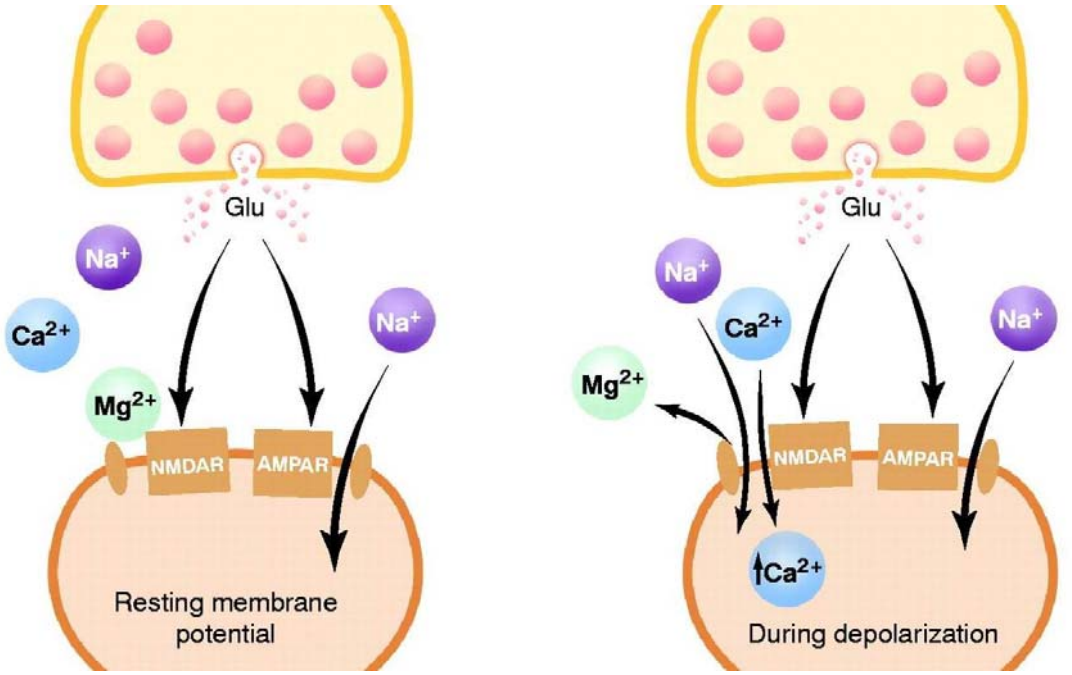

Figure 1.6 NMDAR-dependent LTP. Depolarization of the postsynaptic cell relieves Mg^{2+} block of the NMDAR, allowing Na^{+} and Ca^{2+} to flow into the dendritic spine through the NMDAR. The resultant rise in Ca^{2+} within the dendritic spine is the critical trigger for LTP. Taken from Malenka and Nicoll, 1999.

LTP requires coincident activitity of presynaptic and postsynaptic elements, resulting in a facilitation of transmission. Homosynaptic LTP can be induced experimentally by applying a sequence of short, high-frequency stimulations to the presynaptic cell. Some types of LTP depend on the activation of postsynaptic NMDARs (Fig. 1.6), which result in calcium influx in the postsynaptic cell and the activation of protein kinases, i.e., protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII) (Teyler and DiScenna, 1987; Anwyl, 1989; Madison et al., 1991; Bashir and Collingridge, 1992; Bliss and Collingridge, 1993; Malenka, 1994; Kullmann and Siegelbaum, 1995; Malenka and Nicoll, 1999; Matynia et al., 2002; Malenka and Bear, 2004; Kullmann and Lamsa, 2007). Other types are NMDAR-independent (Johnston et al., 1992; Bear and Malenka, 1994; Bortolotto et al., 1999; Malenka and Bear, 2004; Kullmann and Lamsa, 2007). However, whether the induction of LTP relies exclusively on a postsynaptic process (Matsuzaki et al., 2004; Kerchner and Nicoll, 2008), or rather involves a presynaptic reduction in neurotransmitter release probability (Bayazitov et al., 2007; Ahmed and Siegelbaum, 2009; Enoki et al., 2009), remains under debate (Madison et al., 1991; Malenka, 1994; Isaac et al., 1996; Lisman, 2009).

1.4.2 Long-term depression

The opposite phenomenon of LTP, called long-term depression (LTD), is the weakening of a neuronal synapse that lasts for an extended period of time (minutes to hours *in vitro* and hours to days and months *in vivo*). Cerebellar LTD has been hypothesized to be important for motor learning. Hippocampal LTD may mediate learning, forgetting or behavioural extinction, e.g., the loss of responsiveness to previously effective stimuli. Alternatively, LTD may serve as a homeostatic mechanism to ensure that CNS synapses are not saturated by learning (Ito, 1989; Linden, 1994; Bear and Malenka, 1994; Malenka, 1994; Linden and Connor, 1995; Bear and Abraham, 1996; Debanne and Thompson, 1996; Thiels et al., 1996; Kemp and Bashir, 2001; Debanne et al., 2003; Anwyl,

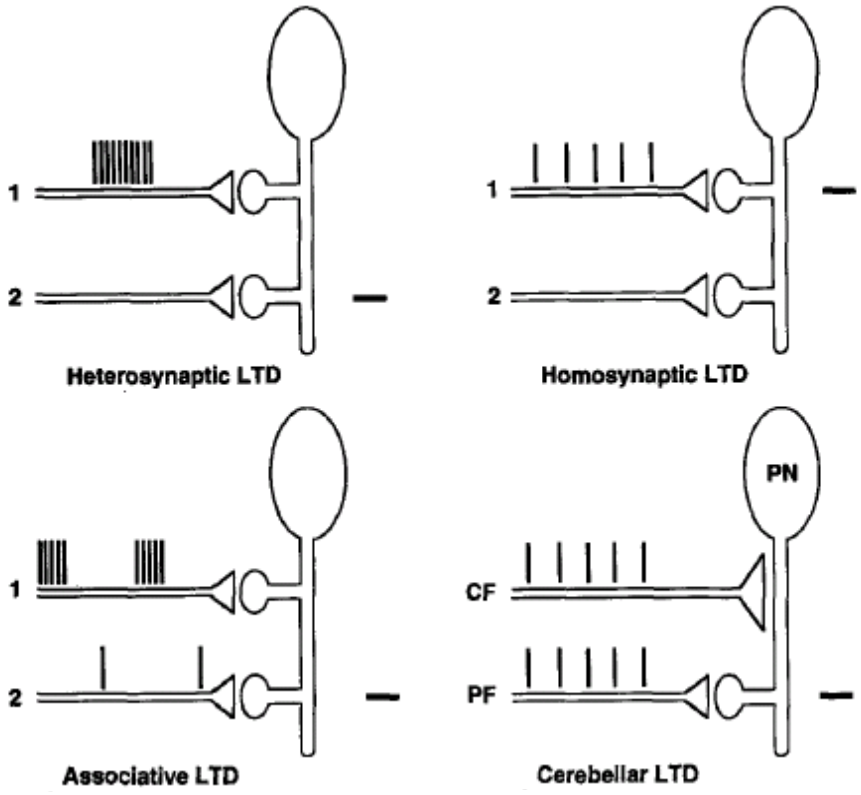

Figure 1.7 Types of LTD. Vertical lines represent stimulating pulses. "—"sign shows the location of LTD. Abbreviations: PN: Purkinje neuron; CF: climbing fiber; PF: parallel fiber. Adatped from Linden, 1994

2006; Cooke and Bliss, 2006; Kullmann and Lamsa, 2007).

There are several types of LTD (Fig. 1.7). In heterosynaptic LTD, inducing stimuli delivered to one pathway result in a depression in the pathway that did not receive stimuli. In homosynaptic LTD, depression is confined to the stimulated pathway. Induction of associative LTD and cerebellar LTD require the activation of two pathways (Linden, 1994; Linden and Connor, 1995; Debanne and Thompson, 1996; Kemp and Bashir, 2001).

The standard protocol for inducing homosynaptic LTD is a long train of low-frequency (1 Hz) stimulation (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Coincidence of pre- and post-synaptic action potentials can induce LTD as well

(Markram et al., 1997). NMDAR-dependent (Bear and Malenka, 1994; Linden and Connor, 1995; Bear and Abraham, 1996; Thiels et al., 1996; Kemp and Bashir, 2001; Malenka and Bear, 2004) and mGluR-dependent (Linden and Connor, 1995; Bear and Abraham, 1996; Bortolotto et al., 1999; Kemp and Bashir, 2001; Malenka and Bear, 2004; Anwyl, 2006) homosynaptic LTD have been characterized.

1.5 Oscillations

1.5.1 Function of oscillations

Network oscillations *in vivo* have been proposed to be important in sensory processing (Laurent and Davidowitz, 1994), in sensory and perceptual binding (Singer and Gray, 1995; Miltner et al., 1999), in motor programming (Murthy and Fetz, 1996), in associative learning (Rodriguez et al., 1999; Buzsáki, 2002), and in epileptogenesis (Grenier et al., 2001, 2003).

1.5.2 Oscillations in hippocampus

In the behaving animals, synchronous oscillations of the membrane potential of large neuronal populations can occur at various frequencies in the hippocampus (Table 1.1).

1.5.3 Methods of inducing oscillation *in vitro*

A variety of population oscillations can be induced in hippocampal slices either by activation of metabotropic glutamate receptors, kainate receptors or muscarinic receptors with drugs or electrical stimulation; or by manipulation of the ionic

Oscillation type	Conditions	Chemical synapses	GJ	Pyramidal cell somata	Interneurons
ING	mGluR + iGluR block	Yes	Yes	—	Each wave
	$[K^+]$ puff + iGluR block	Yes	Yes	—	N.T.
tetanic γ	Elect. stim.	Yes	No?	Variable: can be most beats	Most/all beats; doubles if two-site
				[pyr. cells and interneurons in phase]	
tetanic β	Elect stim. (two-site, strong)	Yes	No?	Skip beats	γ frequency
"pure" VFO	spontaneous or low $[Ca^{2+}]$	No	Yes	Some waves; spikelets	—
persistent γ	kainate, mAChR, mGluR	Yes	Yes	Rare firing	Fire 1/2 to most waves (axo-axonic even more)
				[pyr. cells slightly lead interneurons]	
puff γ	Local hypertonic $[K^+]$	Yes	Yes	Rare firing	N.T.
theta (CA1)	mGluR + AMPA block	Yes	Yes	Rare firing	S. oriens interneurons (O/LM) fire each wave
theta (CA3)	mAChR	Yes (not $GABA_A$)	N.T.	May fire little, or may burst	Probably not necessary

Table 1.1 Properties of *in vitro* hippocampal oscillations. Abbreviations: N.T., not tested; ING: interneuron network gamma; VFO, very fast oscillation (>70 Hz); gamma/beta (15–70 Hz); theta rhythm (4-15 Hz). Taken from Traub et al., 2004

environment (for example, removing of calcium ions); brief oscillations can even occur spontaneously (Traub et al., 2004).

1.5.4 Mechanisms of the generation of theta rhythm

Theta rhythm (4-15 Hz) (Bland 1986; Lopes da Silva et al. 1990; Stewart and Fox

1990; Vinogradova 1995; Vertes and Kocsis 1997; Kamondi et al., 1998; Harris et al., 2001; Buzsáki, 2002) is present mainly during locomotion and other voluntary movements (Grastyán et al., 1959; Vanderwolf, 1969) and rapid eye movement (REM) sleep (Jouvet, 1969). Theta waves have been also assumed to code mnemonic processes (Lisman and Idiart 1995; Raghavachari et al. 2001). Loss of hippocampal theta rhythm results in spatial memory deficits in the rat (Winson 1978).

Mechanisms of the generation of theta rhythm in the hippocampus are not completely understood yet. However, it is clear that the reciprocal connection between the medial septum-diagonal band of Broca (MS-DBB) and the hippocampus plays an essential role (Green and Arduni, 1954; Petsche et al., 1962; Lawson and Bland, 1993; Sik et al., 1994; Markowska et al., 1995; King et al., 1998; Dragoi et al., 1999; Buzsáki 2002; Gulyas et al., 2003). Hippocampal interneurons represent the only output from the hippocampus to the MS-DBB (Toth et al., 1993) and are required for the induction and propagation of theta rhythm (Buzsáki et al., 1983; Sik et al., 1994; Freund and Antal, 1988; Buzsáki and Chrobak, 1995; Cobb et al., 1995; Dragoi et al., 1999; Buzsáki 2002; Gulyas et al., 2003; Mann and Paulsen, 2007). One hippocampal interneuron innervates more than 1,000 pyramidal cells (Halasy et al., 1996). Thus, they are in a key position to synchronize the activity of pyramidal cells.

Chapter 2

Group I metabotropic glutamate receptors in CA1 pyramidal cells are not tonically activated by ambient glutamate in hippocampal slice cultures

2.1 Abstract

Microdialysis experiments report a background level of extracellular glutamate concentration of approximately 2 μM in the brain. The EC_{50} of glutamate for group I mGluRs is ~10 μM. Therefore the group I mGluRs may be tonically activated by the background level of glutamate present in the extracellular space. Here we recorded in CA1 pyramidal neurons and found that the ambient glutamate concentration in the extracellular space is insufficient to tonically activate group I mGluRs in hippocampal organotypic slice cultures.

2.2 Introduction

The concentration of neurotransmitters in the extracellular space of the central nervous system is determined by a balance between release, degradation and uptake mechanisms. Glutamate is released during fast synaptic transmission and its concentration reaches ~1 mM within the synaptic cleft (Clements, 1996). Then glutamate concentration decreases rapidly due to diffusion and uptake by glutamate transporter (Bergles et al. 1999; Attwell and Gibb, 2005). If the release rate is low, in principle, the co-transport of 3 Na^+ and 1 H^+ and the counter-transport of 1 K^+ can lower the extracellular glutamate concentration to ~2 nM (Zerangue and Kavanaugh, 1996; Levy et al., 1998). However, microdialysis experiments report extracellular glutamate concentrations of 0.5-7 μM (typically 2 μM) in the brain *in vivo* and in perfusate from unstimulated brain slices (Johnson 1978; Benveniste et al., 1984; Phillis et al., 1994; Wahl et al., 1994; Bianchi et al. 1999; Meldrum 2000), which are higher than these theoretical values. The EC_{50} of glutamate for NMDA receptors is ~2 μM (Patneau and Mayer, 1990; Hollmann and Heinemann 1994; Meldrum 2000; Nahum-Levy et al., 2001, 2002). Accordingly, tonic activation of NMDA receptors in pyramidal neurons was

observed in acute brain slices (Sah et al., 1989; Angulo et al., 2004; Cavelier and Attwell, 2005; Cavelier et al., 2005; Meur et al., 2007).

The EC_{50} of glutamate for group I mGluRs is ~10 µM (Hollmann and Heinemann 1994; Pin and Duvoisin 1995; Conn and Pin, 1997). Therefore the group I mGluRs may be tonically activated by the background level of glutamate present in the extracellular space. Group I mGluRs modulate NMDA receptor-mediated responses in CA1 pyramidal cells (Aniksztejn et al., 1991, 1992; Harvey and Collingridge, 1993; Contractor et al., 1998; Snyder et al., 2001; Benquet et al., 2002; Zho et al., 2002; Grishin et al., 2004; Ireland and Abraham, 2009). Here we used the activity of NMDA receptors as a sensor to check whether the ambient glutamate concentration in the extracellular space is sufficient to tonically activate group I mGluRs in hippocampal slice cultures.

2.3 Materials and Methods

2.3.1 Slice cultures and electrophysiology

Hippocampal organotypic slice cultures were prepared from 6-day-old Wister rats using the roller-tube techniques, as described previously (Gähwiler et al., 1998). After 3-4 weeks *in vitro* the slice cultures were transferred to a recording chamber with a volume of 1 ml on an upright microscope (Axioskop FS, Zeiss). Slices were superfused at a rate of 1.5 ml/min with artificial cerebrospinal fluid (ACSF, in mM) 137 NaCl, 2.7 KCl, 11.6 $NaHCO_3$, 0.4 NaH_2PO_4, 2.0 $MgCl_2$, 2.8 $CaCl_2$, 5.6 D-glucose and 10 mg/L phenol red (pH 7.4, ~305 mOsm, 33 °C, saturated with 95% O_2 and 5% CO_2).

Whole-cell voltage-clamp recordings were obtained from hippocampal CA1 pyramidal cells with an Axopatch 200B amplifier (Molecular Devices). Recording

pipettes (3-5 MΩ) were filled with (in mM) 120 K-gluconate, 10 L-glutamic acid, 5 KCl, 10 Hepes, 10 EGTA, 2 MgATP, 5 creatine phosphate (CrP), 0.4 NaGTP, 0.07 $CaCL_2$ (pH 7.2, ~290 mOsm). Membrane potentials were corrected for liquid junction potentials. Series resistance (5-15 MΩ) and input resistance were monitored regularly. Currents were filtered at 5 kHz and analyzed off-line (pClamp 9, Molecular Devices). All numerical data are presented as the mean ± SEM. Significance was tested using two-tailed t-test.

2.3.2 Induction of NMDA currents

CA1 pyramidal cells were voltage-clamped at -50 mV. In the presence of 1 μM TTX (sodium channel blocker), 100 μM picrotoxin ($GABA_A$ receptor antagonist) and 10 μM NBQX (AMPA/kainate receptors antagonist), NMDA currents were induced by applying brief pressure pulses (1 bar, ~200 msec) at 40-second intervals to a micropipette filled with NMDA (100 μM) and placed ~100 μm from the soma of the recorded cell.

2.3.3 Drugs

NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide) and (S)-MCPG ((S)-α-methyl-4-carboxyphenylglycine) were purchased from Ascent Scientific (Bristol, UK). All other chemicals were purchased from Sigma/Fluka.

2.4 Results

2.4.1 Group I mGluRs in CA1 pyramidal cells are not tonically activated by ambient glutamate in hippocampal slice cultures

CA1 pyramidal cells were voltage-clamped at -50 mV. In the presence of 1 μM TTX (sodium channel blocker), 100 μM picrotoxin ($GABA_A$ receptor antagonist) and 10 μM NBQX (AMPA/kainate receptors antagonist), NMDA currents were induced by applying brief pressure pulses (1 bar, ~200 msec) at 40-second intervals to a micropipette filled with NMDA (100 μM) and placed ~100 μm from the soma of the recorded cell (Fig. 2.1A). Application of the group I mGluRs antagonist (S)-MCPG did not modulate NMDA receptor-mediated responses in CA1 pyramidal cells (500 μM; 102 ± 3% of baseline, n = 12, p = 0.8; Fig. 2.1B). In other words, NMDA receptors were not tonically modulated by group I mGluRs. MCPG did not change the holding current of pyramidal cells voltage-clamped at -50 mV (n = 12, p = 0.5), indicating that there was no tonic current mediated by group I mGluRs. Take together, we conclude that group I mGluRs in CA1 pyramidal cells are not tonically activated by the background level of glutamate present in the extracellular space.

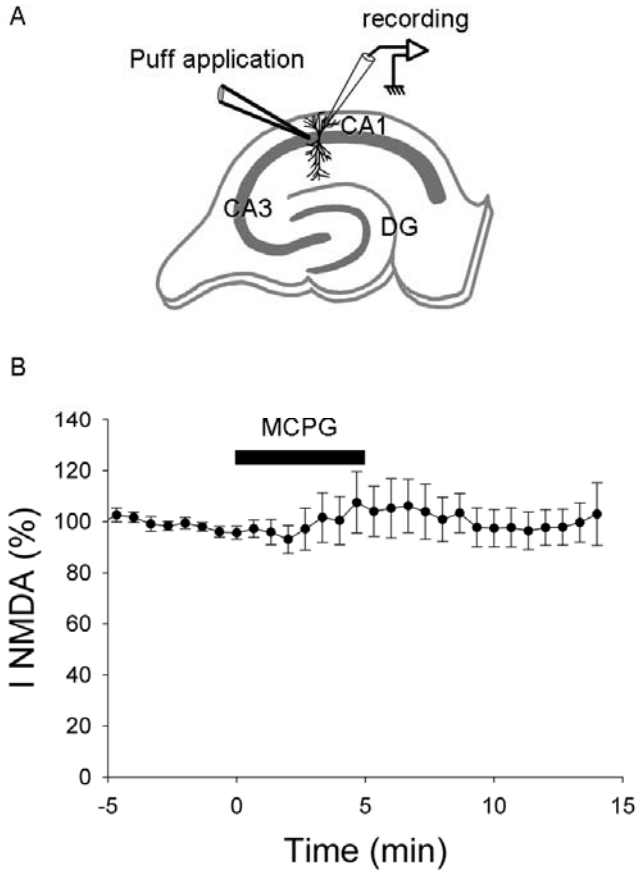

Figure 2.1 Group I metabotropic glutamate receptors in CA1 pyramidal cells are not tonically activated by ambient glutamate in hippocampal slice cultures. (A) Schematic drawing of induction of NMDA currents. (B) Application of 500 μM (S)-MCPG did not modulate NMDA receptor-mediated responses in CA1 pyramidal cells (n = 12).

2.5 Discussion

This study shows that there is no tonic activation of group I mGluRs in CA1 pyramidal cells in hippocampal organotypic slice cultures.

Microdialysis experiments report a background level of extracellular glutamate concentration of 0.5-7 μM (typically 2 μM) in the brain *in vivo* and in perfusate from unstimulated brain slices (Johnson 1978; Benveniste et al., 1984; Phillis et al., 1994; Wahl et al., 1994; Bianchi et al. 1999; Meldrum 2000). Our experiments are performed in hippocampal organotypic slice cultures, in which the background level of extracellular glutamate may be lower than in the brain *in vivo* or in acute brain slices. Therefore a tonic activation of group I mGluRs cannot be observed in hippocampal organotypic slice cultures.

A recent study shows that the ambient concentration of glutamate in acute hippocampal slices is ~25 nM (Herman and Jahr, 2007), 100-fold lower than former reports (Johnson 1978; Benveniste et al., 1984; Phillis et al., 1994; Wahl et al., 1994; Bianchi et al. 1999; Meldrum 2000) and is inconsistent with the reports of tonic activation of NMDA receptors (Sah et al., 1989; Angulo et al., 2004; Cavelier and Attwell, 2005; Cavelier et al., 2005; Meur et al., 2007). However, it may explain why tonic activation of group I mGluRs is not observed in my study.

Consistent with the above result, in chapter 3, we find that multiple Shaffer collateral inputs are required to activate group I mGluRs in CA1 pyramidal cells.

Chapter 3

Activation conditions for the induction of metabotropic glutamate receptor-dependent LTD in hippocampal CA1 pyramidal cells

3.1 Abstract

Two forms of homosynaptic long-term depression (LTD) are distinguished in hippocampal CA1 pyramidal cells, one which is NMDA receptor-dependent and the other metabotropic glutamate receptor (mGluR)-dependent. Although the molecular processes involved in mGluR-LTD are well characterized, the conditions of circuit activation required for its induction remain unclear. We show that mGluR-LTD cannot be induced in synaptically coupled CA3-CA1 pyramidal cell pairs. Experiments to address the underlying mechanisms indicate that even when glutamate transporters are blocked, one presynaptic cell releases insufficient glutamate to evoke an mGluR-mediated current in a connected CA1 cell. These findings imply that extrasynaptic diffusion is not a limiting factor, and are consistent with a sparse distribution of functional mGluRs in the dendritic tree of pyramidal cells. Thus, the discharge of multiple Schaffer collaterals to a targeted cell is necessary for mGluR-LTD. Our experiments indicate that approximately eight CA3 inputs to a CA1 pyramidal cell must be activated to induce mGluR-LTD.

3.2 Introduction

Homosynaptic LTD in the hippocampus is well characterized as an NMDA receptor-dependent form of synaptic plasticity (NMDAR-LTD), as first described at the Schaffer collateral input to CA1 pyramidal cells (Dudek and Bear, 1992; Mulkey and Malenka, 1992). An additional form of LTD at Schaffer collateral synapses onto CA1 pyramidal cells that requires activation of mGluRs (mGluR-LTD) was also reported (Stanton et al., 1991; Bolshakov and Siegelbaum, 1994;

Oliet et al., 1997). Significant progress has been made in understanding the induction mechanisms and intracellular transduction pathways involved in each form (Kemp and Bashir, 2001; Anwyl, 2006). Both appear to be induced postsynaptically (Oliet et al., 1997), but whether their expression relies exclusively on postsynaptic processes (Snyder et al., 2001; Xiao et al., 2001; Zhou et al., 2004; Zhang et al., 2006; Brager and Johnston, 2007), or rather involves presynaptic reduction in neurotransmitter release probability (Bolshakov and Siegelbaum, 1994; Fitzjohn et al., 2001; Watabe et al., 2002; Enoki et al., 2009), remains under debate (Lisman, 2009). For the induction of NMDAR-LTD, it has been shown that cooperative interactions of multiple presynaptic inputs are not essential, such that appropriately timed discharge of a single CA3 pyramidal cell can lead to homosynaptic LTD in a targeted CA1 pyramidal cell (Debanne et al., 1996). A corresponding investigation of the circuit requirements for induction of mGluR-LTD is at present lacking. Here we recorded from synaptically connected pairs of CA3 and CA1 hippocampal pyramidal cells in organotypic slice cultures, and find that in contrast to NMDA-LTD, multiple Schaffer collateral input is required for the induction of mGluR-LTD in a targeted CA1 pyramidal cell.

3.3 Materials and Methods

3.3.1 Slice cultures and electrophysiology

For the preparation of hippocampal organotypic slice cultures, see chapter 2.

Recordings were obtained from hippocampal pyramidal cells with an Axopatch 200B amplifier (Molecular Devices). Both low resistance (3-5 MΩ) and high resistance (10-15 MΩ) recording pipettes were used, which were filled with (in

mM) 120 K-gluconate, 10 L-glutamic acid, 5 KCl, 10 Hepes, 1 EGTA, 2 MgATP, 5 creatine phosphate (CrP), 0.4 NaGTP, 0.07 $CaCL_2$ (pH 7.2, ~290 mOsm). For paired recordings, the postsynaptic pipette was filled with (in mM) 126.6 CsF, 8.4 CsCl, 10 Hepes, 10 EGTA, 2 MgATP, 5 creatine phosphate, 0.4 NaGTP (pH 7.2, ~290 mOsm). Membrane potentials were corrected for liquid junction potentials. Signals were filtered at 5 kHz and analyzed off-line (pClamp 9, Molecular Devices). All numerical data are presented as the mean ± SEM. Significance was tested using two-tailed t-test.

3.3.2 Extracellular stimulation

For monopolar extracellular stimulation of the Schaffer collaterals, glass pipettes filled with ACSF were placed in the stratum radiatum, 100-200 μm away from the recorded cell. For the experiments depicted in supplemental Fig. 3, the monopolar stimulation pipette was positioned in CA3 stratum oriens to ensure the selective activation of Schaffer collaterals. LTD was induced with a standard low frequency stimulation (LFS) protocol (1 Hz, 900 pulses, 100 μs duration; Dudek and Bear, 1992; Manahan-Vaughan, 1997; Wang et al., 2008), or with a paired pulse low frequency stimulation (PP-LFS) protocol (1 Hz, 900 pairs of pulses, 50 ms inter-stimulus interval, 100 μs duration; Kemp and Bashir, 1997) in hippocampal CA1 pyramidal cells voltage-clamped at -70 mV in the presence of the NMDAR antagonist D-AP5 (50 μM). The stimulation intensity was 10-20 μA to induce a response of 50-70% of the maximum. To evoke a current mediated by group I mGluRs, high frequency stimulation (100 Hz, 1-4 pulses, 10-50 μA, 100 μs duration) was applied. CA1 pyramidal cells were voltage-clamped at -50 mV. AMPA, NMDA, $GABA_A$ and $GABA_B$ receptors were blocked by CNQX (50 μM), D-AP5 (100 μM), picrotoxin (100 μM) and CGP 62349 (5 μM), respectively.

3.3.3 Perforated patch-clamp

Recording pipettes (3-5 MΩ) were filled with (in mM) 120 K-gluconate, 10 L-glutamic acid, 5 KCl, 10 Hepes, 1 EGTA, 2 MgATP, 5 creatine phosphate, 0.4 NaGTP, 2.5 $CaCL_2$ (pH 7.2, ~290 mOsm). Stock solution of amphotericin B (60 mM) was prepared in DMSO and added to the pipette solution to a final concentration of 0.3 mM. A fresh solution was prepared every 1.5 h, sonicated 5-10 min and kept in the dark.

3.3.4 Drugs

CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), D-AP5 (D-(-)-2-amino-5-phosphonopentanoic acid) and (S)-MCPG ((S)-α-methyl-4-carboxyphenylglycine) were purchased from Ascent Scientific (Bristol, UK). DL-TBOA (DL-threo-b-benzyloxyaspartic acid) was purchased from Tocris (Bristol, UK). CGP 62349 was kindly provided by Novartis (Basel, Switzerland). All other chemicals were purchased from Sigma/Fluka.

3.4 Results

3.4.1 A single CA3 pyramidal cell does not induce mGluR-LTD in a connected CA1 pyramidal cell

In the presence of D-AP5 (50 μM), a standard LTD protocol evoking a low frequency train of action potentials in a CA3 pyramidal cell (1 Hz, 900 APs) failed to induce mGluR-LTD in a synaptically connected CA1 pyramidal cell in hippocampal slice cultures (98 ± 4% of baseline, n = 6, p = 0.5, recording pipette resistance: 3-5 MΩ; Fig. 3.1A; Fig. 3.2). Because certain forms of synaptic plasticity are disrupted by cytoplasmic washout (Kullmann and Lamsa, 2007), this experiment was repeated using high resistance recording pipettes (10-15 MΩ). Again, LTD was not observed (98 ± 3% of baseline, n = 3, p = 0.6; Fig. 3.1A). Paired-pulse low frequency stimulation (PP-LFS) represents a more efficient protocol for the induction of mGluR-LTD (Kemp and Bashir, 1997). However, even PP-LFS (1 Hz, 900 paired APs, 50 ms inter-stimulus interval, 10-15 MΩ for the CA1 electrode) did not induce mGluR-LTD in CA3-CA1 connected cell pairs (99 ± 4% of baseline, n = 4, p = 0.6; Fig. 3.1B). As postsynaptic mGluRs in hippocampal pyramidal cells are localized in perisynaptic and extrasynaptic zones (Lujan et al., 1996), membrane transporters may prevent glutamate from accessing these receptors. But even in the presence of TBOA (10 μM), a glutamate transporter blocker (Jabaudon et al., 1999), LTD was not induced (102 ± 6% of baseline, n = 3, p = 0.8; Fig. 3.1B). Taken together, these results suggest that activity in a single CA3 pyramidal cell is insufficient to induce mGluR-LTD in a targeted CA1 pyramidal cell. In contrast, when Schaffer collaterals were stimulated with an extracellular electrode (LFS, stimulation intensity: 10-20 μA), thereby activating several CA3 pyramidal cell axons, mGluR-LTD was consistently observed (70 ± 3% of baseline, n = 8, p < 0.001; Fig. 3.1C). Interestingly, mGluR-LTD induced by extracellular stimulation was significantly enhanced when recording with high resistance pipettes (42 ± 3% of

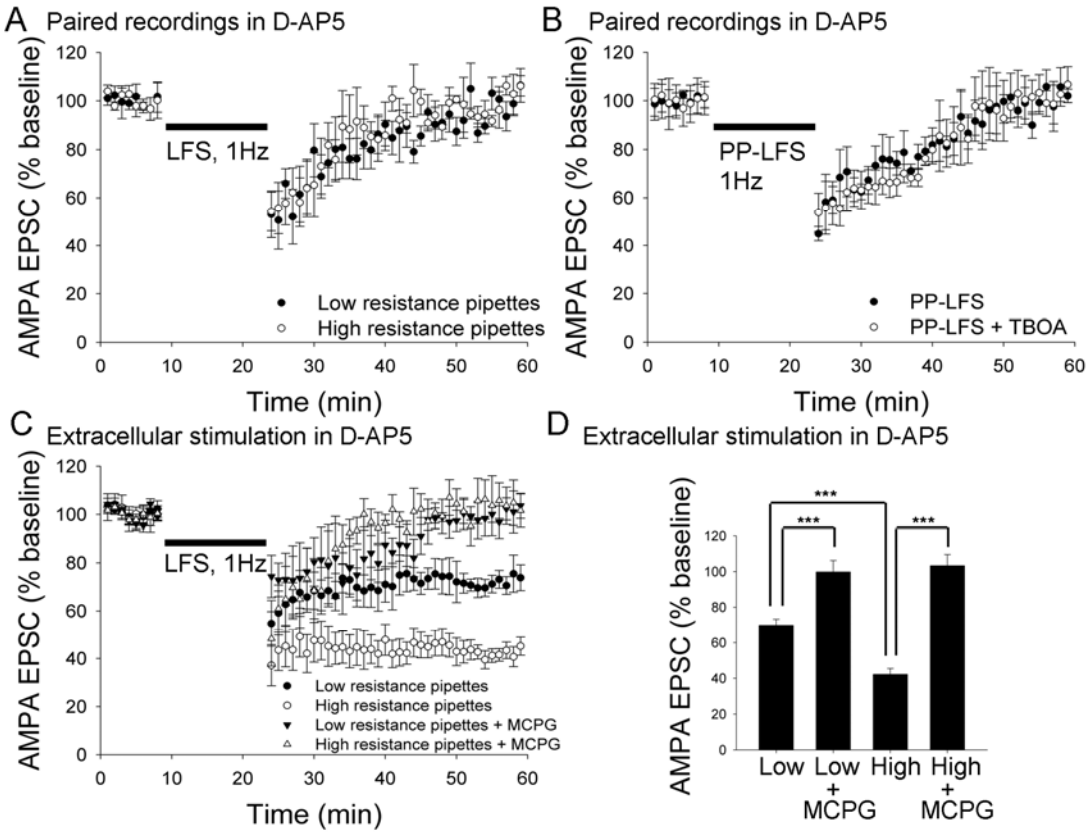

Figure 3.1 Activation of a single CA3 pyramidal cell is insufficient to induce mGluR-LTD in a synaptically connected CA1 pyramidal cell. (A) In the presence of D-AP5, a low frequency train of action potentials (1 Hz, 900 APs) in a CA3 cell did not induce mGluR-LTD in a CA1 cell (n = 6), even when high resistance recording pipettes were used to minimize dialysis (n = 3). (B) A low frequency train of paired action potentials (PP-LFS) also failed to induce mGluR-LTD (n = 4) even when glutamate transporters were blocked with TBOA (n = 3). (C) mGluR-LTD can be induced in a CA1 pyramidal cell with extracellular stimulation of Schaffer collaterals (1 Hz, 900 pulses, 10-20 μA, in D-AP5). The amplitude of mGluR-LTD was significantly greater when high resistance pipettes were employed (n = 8 and 5 for low and high resistance pipettes, respectively, $p < 0.001$). 500 μM (S)-MCPG prevented mGluR-LTD (n = 5). (D) Summary of results from extracellular stimulation experiments. ***$p < 0.001$.

baseline, n = 5, $p < 0.001$, high versus low resistance pipettes: $p < 0.001$; Fig. 3.1D), indicating that cytoplasmic dialysis significantly disrupts metabotropic signaling. Application of (S)-MCPG (500 μM) prevented mGluR-LTD (low resistance pipettes: 100 ± 4% of baseline, n = 5, $p = 0.95$; high resistance

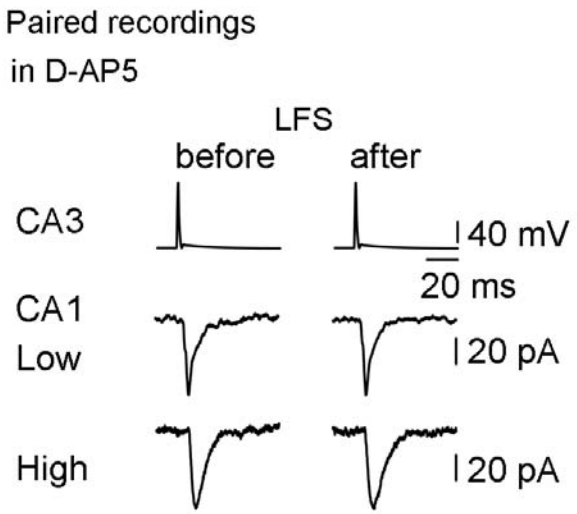

Figure 3.2 Representative traces obtained during paired recordings for the experimental data plotted in Figure 3.1A. Traces represent averages of 10 sweeps.

pipettes: 103 ± 6%, n = 5, p = 0.6; Fig. 3.1C). In the relatively young slice cultures used here (3-4 weeks), the PP-LFS protocol did not further enhance mGluR-LTD (n = 5, p = 0.98), and blocking glutamate transporters with TBOA had no significant effect (n = 5, p = 0.4; Fig. 3.3).

When Schaffer collaterals are stimulated extracellularly, it can be difficult to rule out that the perforant pathway, other CA1 pyramidal cells, or subicular cells are inadvertently activated. In our slice cultures, however, the subiculum is removed, and perforant path fibers have completely disappeared after two weeks in culture (Zimmer and Gähwiler, 1984). Furthermore, monosynaptic connectivity between CA1 pyramidal cells is 0.8% in acute slices (Thomson & Radpour, 1991), and marginally higher in slice cultures. Nevertheless, we performed experiments in which Schaffer collaterals were activated with the stimulating electrode positioned in the stratum oriens instead of the stratum radiatum of CA3 (n = 4), making it unlikely that CA1 pyramidal cell axons are activated. The data show that mGluR-LTD was not significantly different (p = 0.9; stratum oriens: 43 ± 4% of baseline, n = 4; stratum radiatum: 42 ± 3% of baseline, n = 5; Fig. 3.4).

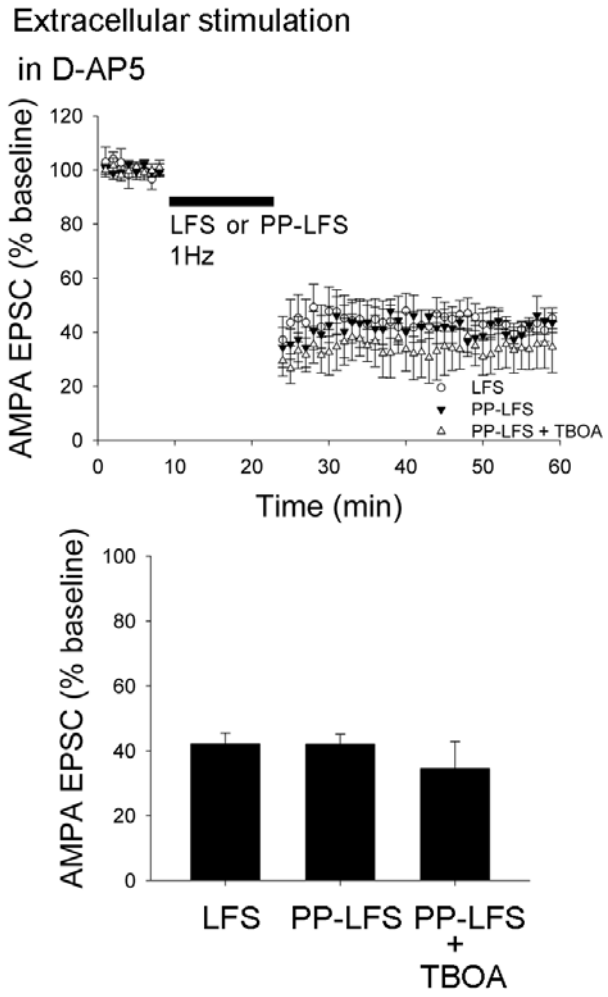

Figure 3.3 A comparison of mGluR-LTD induced in CA1 pyramidal cells recorded with high resistance pipettes by extracellular stimulation of Schaffer collaterals using single pulse LFS, PP-LFS, and PP-LFS in the presence of 10 μM TBOA (n = 5 for each protocol). No significant difference in the amplitudes of LTD were observed (LFS: 42 ± 3% of baseline, $p < 0.001$; PP-LFS: 42 ± 3% of baseline, $p < 0.001$; PP-LFS + TBOA: 35 ± 8% of baseline, $p < 0.001$; LFS versus PP-LFS: $p = 0.98$; PP-LFS versus PP-LFS + TBOA: $p = 0.4$).

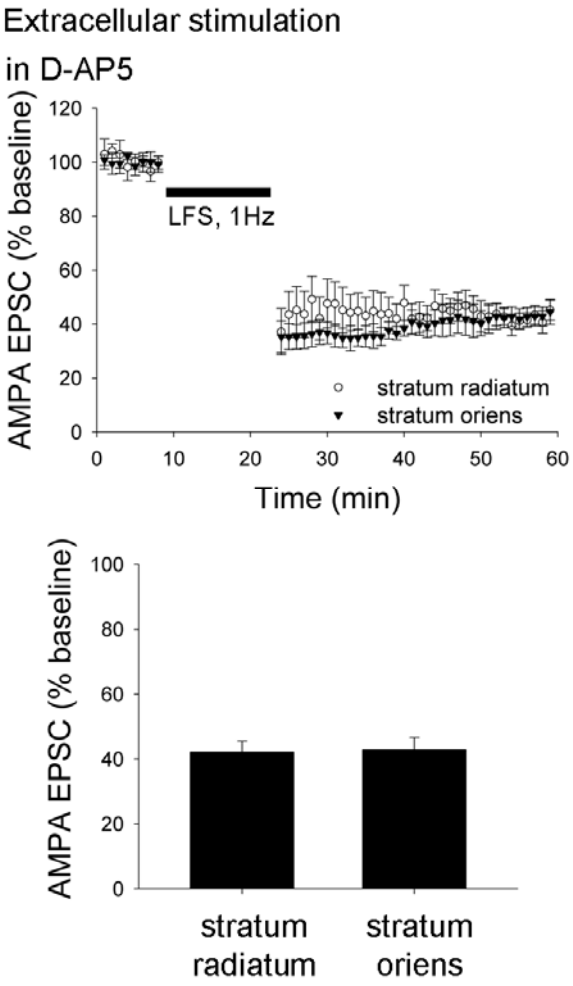

Figure 3.4 Evidence that a stimulation electrode positioned in CA3 stratum radiatum activates Schaffer collaterals but not other excitatory inputs to CA1. The magnitude of LTD induced by activating Schaffer collaterals with a stimulating placed in CA3 stratum oriens (where CA1 pyramidal cell axons are unlikely to be activated) is not significantly different than when the stimulating electrode is placed in stratum radiatum ($p = 0.9$; $n = 4$ and 5 for stratum oriens and stratum radiatum, respectively).

3.4.2 A single CA3 pyramidal cell does not evoke a somatic current mediated by mGluRs in a connected CA1 pyramidal cell

To examine why mGluR-LTD could not be induced between pyramidal cell pairs, we checked whether an mGluR-mediated current could be evoked in a CA1 pyramidal cell in response to firing of a single connected CA3 pyramidal cell. Following blockade of NMDA, AMPA/kainate, $GABA_A$ and $GABA_B$ receptors, even a high frequency train of 30 APs at 100 Hz failed to evoke a metabotropic response in a CA1 pyramidal cell recorded with a high resistance pipette at -50 mV, a membrane potential where mGluR-mediated currents are maximal (Gee et al., 2003) (n = 3; Fig. 3.5A, lower). Blocking glutamate transporters (TBOA 50 μM) also did not reveal a metabotropic response (n = 3; Fig. 3.5A, lower). A similar negative result was obtained with the perforated patch-clamp method (n = 3; Fig. 3.6A).

With extracellular stimulation of Schaffer collaterals (100 Hz, 4 pulses, 20-50 μA, 100 μs duration), mGluR-mediated EPSCs were consistently evoked in CA1 pyramidal cells, as previously reported (Congar et al., 1997; Nakamura et al., 1999). A metabotropic response was, however, only observed with high resistance recording pipettes (n = 10 and 28 for low and high resistance pipettes, respectively; Fig. 3.5B, upper). The time to peak current (933.3 ± 67.6 ms, n = 28) and the 90-10% decay time (2927.2 ± 187.0 ms, n = 28) of these EPSCs were two orders of magnitude greater than those of ionotropic AMPA EPSCs. Consistent with responses mediated by group I mGluRs, the EPSCs were blocked by (S)-MCPG (500 μM, n = 13; Fig. 3.5B, lower). Furthermore, TBOA (10 μM) significantly potentiated mGluR EPSCs evoked by extracellular stimulation (192 ± 24% of baseline, n = 6, $p < 0.05$; Fig. 3.7). Similar results were obtained with the perforated patch-clamp method, with no significant difference in response amplitude obtained with high resistance pipettes (n = 8) versus perforated patch-clamp (n = 5, $p > 0.3$; Fig. 2C; Fig. 3.6B).

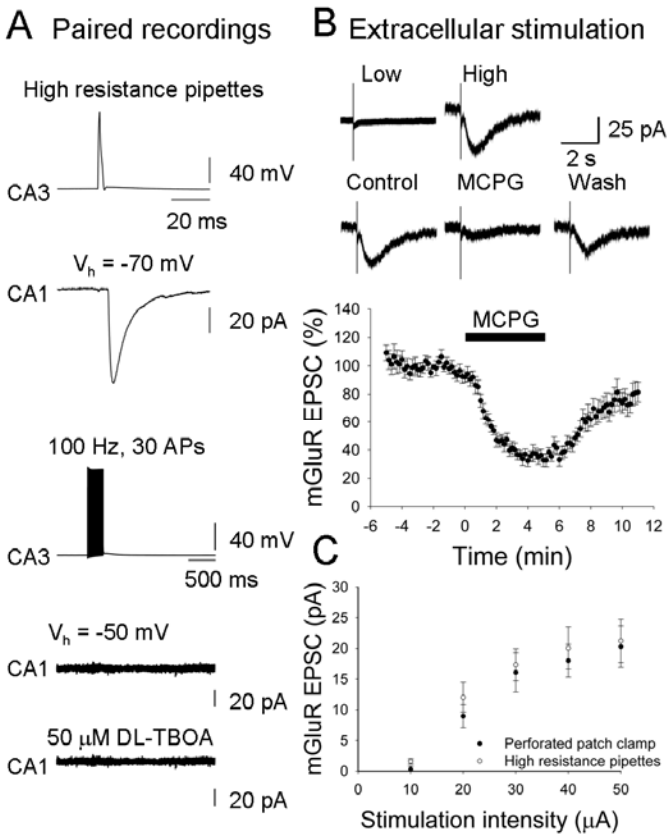

Figure 3.5 Activation of a single CA3 pyramidal cell does not evoke a discernible somatic current mediated by mGluRs in a synaptically connected CA1 pyramidal cell. (A) To enhance mGluR-mediated currents, postsynaptic CA1 cells were voltage-clamped at -50 mV using recording pipettes with a resistance of 10-15 MΩ. Upper, a single AP in a CA3 pyramidal cell elicited a fast EPSC in a CA1 pyramidal cell (V_h = -70 mV). Lower, blocking NMDA (50 μM D-AP5), AMPA/kainate (50 μM CNQX), $GABA_A$ (100 μM picrotoxin), and $GABA_B$ receptors (5 μM CGP 62349) failed to reveal a slow metabotropic current in response to a 100 Hz train of 30 APs. Inhibition of glutamate transporters (50 μM TBOA) under these conditions also did not reveal an mGluR-mediated current. Traces represent averages of 10 sweeps. (B) Upper, extracellular stimulation of Schaffer collaterals (100 Hz, 4 pulses, 30 μA, V_h = -50 mV) did not evoke an mGluR-mediated response in CA1 pyramidal cells when recording pipettes of 5 MΩ were used. The same stimulation evoked a slow EPSC when using high resistance recording pipettes (12 MΩ). Lower, the slow EPSC was inhibited by (S)-MCPG (500 μM, n = 13). Responses were elicited at 0.1 Hz. Traces represent averages of 5 sweeps. (C) Average amplitudes of mGluR EPSCs evoked with extracellular stimulation (n = 8 and 5 for high resistance pipettes and perforated patch-clamp, respectively).

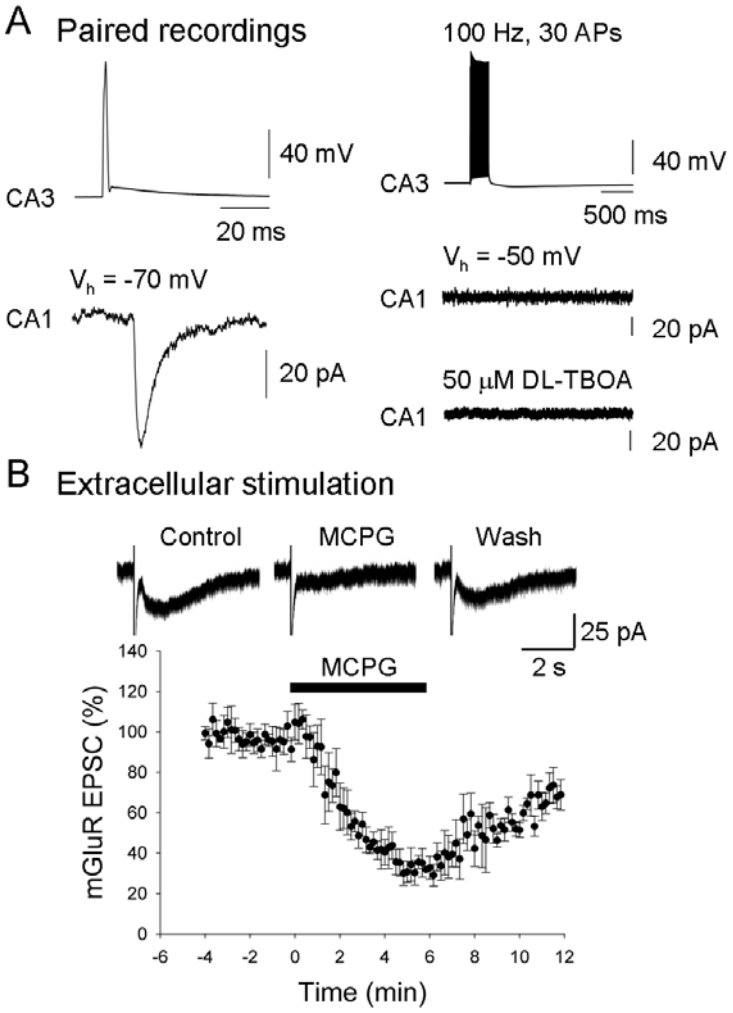

Figure 3.6 Perforated patch-clamp data showing that activation of a single CA3 pyramidal cell does not evoke a current mediated by mGluRs in a synaptically connected CA1 pyramidal cell. (A) Triggering one AP in a CA3 pyramidal cell by current injection elicited an EPSC in a CA1 pyramidal cell (V_h = -70 mV). (B) In the presence of CNQX (50 μM), D-AP5 (50 μM), picrotoxin (100 μM), and CGP 62349 (5 μM), triggering 30 APs at 100 Hz in a CA3 pyramidal cell elicited no current in the connected CA1 pyramidal cell (V_h = -50 mV), even when glutamate transporters were blocked with 50 μM TBOA. Traces represent averages of 10 sweeps. (C) Extracellular stimulation of the Schaffer collaterals evoked a slow EPSC mediated by mGluRs in a CA1 pyramidal cell. The mGluR EPSC amplitude was inhibited by (S)-MCPG (500 μM, n = 4). Responses were elicited at 0.1 Hz. Traces represent averages of 5 sweeps.

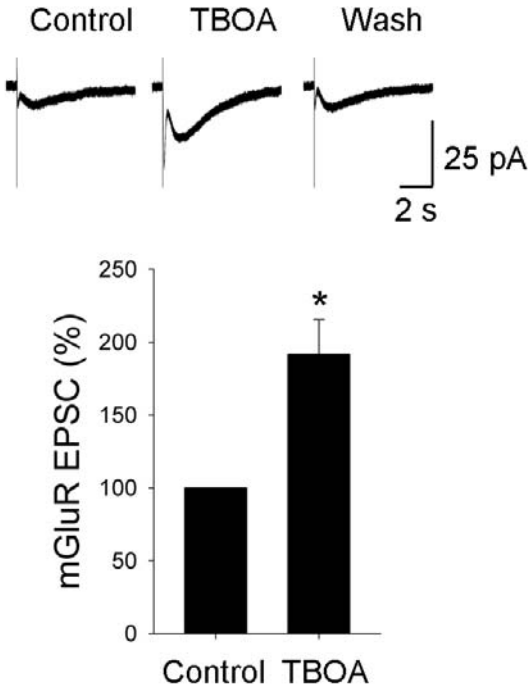

Figure 3.7 Application of TBOA potentiated mGluR EPSCs evoked by extracellular stimulation (10 μM; n = 6). Recording pipettes with resistance of 10-15 MΩ were employed. *p < 0.05. Traces represent averages of 5 sweeps.

3.4.3 Estimating the number of Schaffer collaterals required to induce mGluR-mediated responses

As stimulation of a single CA3 pyramidal cell did not induce a metabotropic current in a CA1 pyramidal cell, we performed experiments to estimate the minimum number of synaptic inputs to a CA1 pyramidal cell that must be activated to evoke an mGluR-mediated response. We determined that a threshold stimulation intensity of 20 μA applied to the Schaffer collaterals (100 Hz,

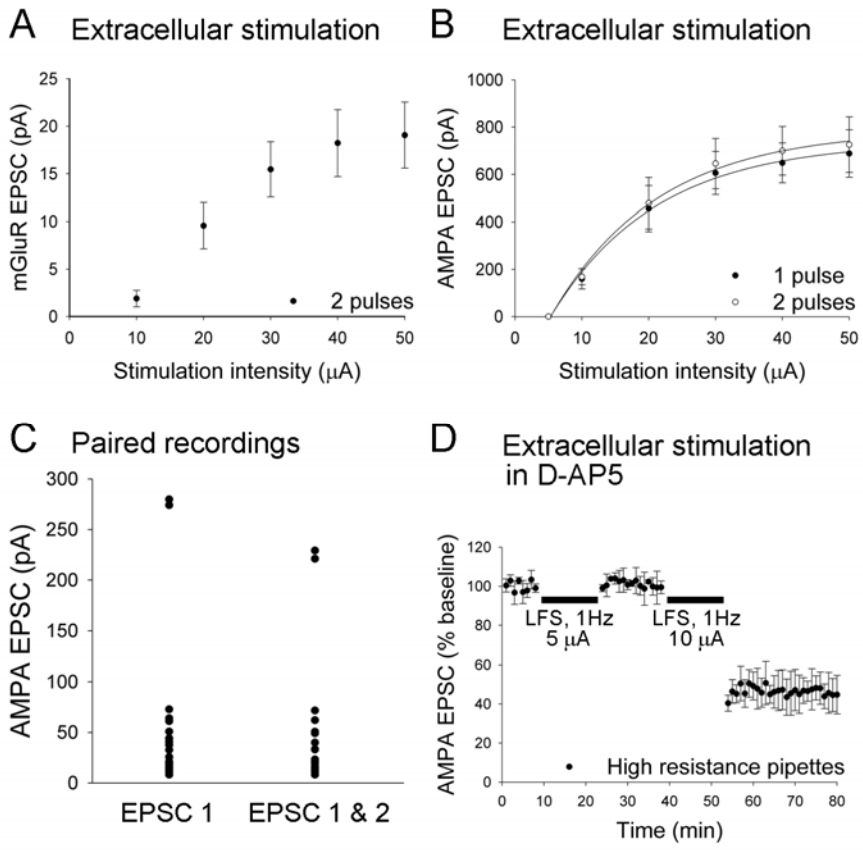

Figure 3.8 Estimating the number of CA3 axons targeting one CA1 pyramidal cell that must be activated to induce an mGluR-mediated current or mGluR-LTD. (A) Average amplitudes of mGluR EPSCs evoked with 2 pulses at 100 Hz were plotted against stimulation intensity (10-50 μA, V_h = -50 mV, n = 5, high resistance pipettes). (B) AMPA EPSCs in CA1 pyramidal cells elicited with extracellular stimulation of Schaffer collaterals with 1 or 2 pulses at 100 Hz (V_h = -70 mV). Average amplitudes of AMPA EPSCs were plotted against stimulation intensity (n = 7). (C) Paired recordings between CA3-CA1 pyramidal cells. Four APs (100 Hz) triggered in a CA3 pyramidal cell elicited AMPA EPSCs in a connected CA1 pyramidal cell voltage-clamped at -70 mV. Amplitudes of the first EPSCs and the average amplitudes of the first and second EPSCs are shown (n = 22). Outliers may reflect multiquantal release (Tong and Jahr, 1994). (D) mGluR-LTD can be induced in a CA1 pyramidal cell with extracellular stimulation of Schaffer collaterals (1 Hz, 900 pulses, in 50 μM D-AP5) with a stimulation intensity of 10 μA, but not 5 μA (n = 3). The amplitude of the AMPA response, evoked with a stimulation intensity corresponding to the threshold to induce mGluR-LTD or an mGluR-mediated EPSC, was divided by the amplitude of a unitary AMPA response to estimate the number of inputs to a CA1 pyramidal cell required for an mGluR-mediated response.

2 pulses,100 μs duration) is necessary to evoke a discernible mGluR-mediated current of ~10 pA in CA1 pyramidal cells voltage-clamped at -50 mV (n = 5; Fig. 3.8A; Fig. 3.9A). The identical 2-pulse stimulation protocol evoked two AMPA EPSCs, whose mean amplitude was ~470 pA at 20 μA, derived from the exponential fit of the curve (f = y0+a*(1-exp(-b*x)); Fig. 3.8B; Fig. 3.9B). As the median amplitude of the two unitary AMPA responses was 23.1 pA (n = 22 pairs; Fig. 3.8C; Fig. 3.9C), we can estimate that ~20 CA3 synaptic inputs released glutamate to yield a response of 470 pA. Therefore, approximately 20 CA3 pyramidal cell axons targeting a given CA1 pyramidal cell must be activated concurrently to induce a somatic current mediated by group I mGluRs. We then checked whether the threshold stimulation intensity for mGluR-LTD is similar to that required to induce an mGluR-mediated current. We found that a much lower stimulation intensity of 10 μA (at 1 Hz) was sufficient to induce mGluR-LTD. At a still lower stimulation intensity of 5 μA, an AMPA receptor-mediated current was no longer observed. As glutamate binds with an approximately 30-fold higher affinity to mGluRs (Conn and Pin, 1997) than to AMPA receptors (Jonas and Sakmann, 1992), it is nonetheless possible that this lower stimulation intensity might induce mGluR-LTD. However, when stimulation intensity was reduced to 5 μA, LTD was no longer observed (5 μA: 101 ± 4% of baseline, p = 0.9; 10 μA: 46 ± 7% of baseline, p < 0.05; n = 3; Fig. 3.8D). Thus, an intensity of 10 μA is close to threshold for inducing mGluR-LTD. A stimulus of 10 μA evokes an extracellular AMPA receptor-mediated response of ~190 pA (Fig. 3.8B), which when divided by the median value of a single unitary AMPA response (23.3 pA, n = 22 pairs; Fig. 3.8C) provides an estimate of 8 active CA3 inputs required to induce mGluR-LTD in a targeted CA1 pyramidal cell.

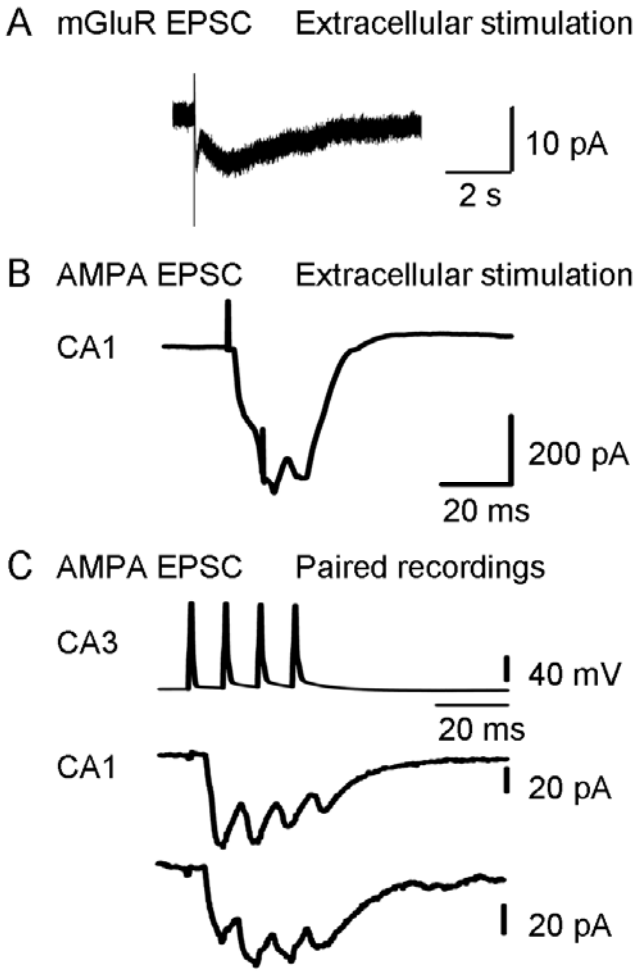

Figure 3.9 Representative traces obtained during recordings for the experimental data plotted in Fig. 3.8. (A) an mGluR EPSC evoked by extracellular stimulation of Schaffer collaterals (100 Hz, 2 pulses, 20 μA, V_h = -50 mV). (B) AMPA EPSC evoked by extracellular stimulation of Schaffer collaterals (100 Hz, 2 pulses, 20 μA, V_h = -70 mV). (C) AMPA EPSCs in paired recordings between CA3-CA1 pyramidal cells. Traces from two different pairs are shown. Four APs (100 Hz) in a CA3 pyramidal cell elicited unitary AMPA EPSCs in a connected CA1 pyramidal cell voltage-clamped at -70 mV. Traces represent averages of 5, 5 and 20 sweeps in (A), (B) and (C), respectively.

3.5 Discussion

The main finding of this study is that in contrast to NMDAR-LTD (Debanne et al., 1996), mGluR-LTD assessed by somatic recording in CA1 pyramidal cells could not be induced by stimulating a single synaptically connected CA3 pyramidal cell. A number of possibilities may account for this difference. First, NMDA receptors in CA1 pyramidal cells are usually located in the middle of the synaptic disk (Racca et al., 2000), whereas metabotropic receptors display a perisynaptic or extrasynaptic localization (Lujan et al., 1996). Thus, cooperative presynaptic activity could be necessary to allow pooling and diffusion of neurotransmitter to the more distantly positioned metabotropic receptors, as demonstrated for activation of $GABA_B$ receptors (Scanziani, 2000). This mechanism, however, does not appear to be involved in inducing mGluR current, as inhibition of glutamate transporters with TBOA to promote synaptic spillover of neurotransmitter did not reveal a response. A second possibility is that as opposed to NMDA receptors, which are expressed in virtually all spines in CA1 pyramidal cells, (Takumi et al., 1999; Racca et al., 2000), the expression of dendritic mGluRs may be relatively low, such that diffusion of transmitter from a single release site will rarely reach an mGluR-positive spine. Evidence for a sparse distribution of functional dendritic mGluRs is provided in a recent study showing that metabotropic responses in CA1 pyramidal cells could only be induced in a subset of endoplasmic reticulum-containing mushroom spines (Holbro et al., 2009), which represent less than 10% of all spines. Furthermore, the failure rate with glutamate uncaging for mGluR-mediated responses in mGluR-positive spines was approximately 80%. Even though a single CA3 pyramidal cell can make multiple functional contacts with a synaptically coupled CA1 pyramidal cell (Hsia et al., 1998) – estimated at between 2 to 4 (Sorra and Harris, 1993) – the probability that one of these few contacts will produce a metabotropic response is therefore very low. Indeed, our experiments indicate that ~20 CA3 pyramidal cell inputs to a CA1 pyramidal cell must be activated for reliable induction of somatic mGluR-mediated current. This is, nevertheless, a

relatively small number considering that a CA1 pyramidal cell *in vivo* receives, on average, input from 5,500 CA3 pyramidal cells (Amaral et al., 1990). Moreover, we estimate that even fewer CA3 inputs (less than ten) must be activated to induce mGluR-LTD. When an mGluR-positive spine is first identified and then specifically stimulated by two-photon glutamate uncaging, it is, however, possible to obtain mGluR-LTD (Holbro et al., 2009). This difference to our experiments is likely to reflect the fact that prior identification of mGluR-positive synapses is not possible at present with paired recording, and, as there are multiple functional contacts between CA3-CA1 pyramidal cell pairs, LTD of at best one of these synapses may not be discernible within the compound synaptic response. Furthermore, localized uncaging of glutamate may be more efficient than synaptically released glutamate at reaching peripherally localized mGluRs.

A lower threshold for mGluR-LTD as compared to mGluR-mediated current in CA1 pyramidal cells could reflect differences in the respective intracellular transduction mechanisms. Thus, an mGluR-dependent calcium signal to initiate LTD induction may have a lower stimulation threshold than an mGluR-mediated current, as demonstrated in cerebellar Purkinje cells (Finch and Augustine, 1998; Takechi et al., 1998). It has not been possible to repeat this type of experiment in CA3-CA1 paired recordings because of the difficulty in localizing the very few activated spines in the large dendritic tree of a pyramidal cell.

A second finding emerging from our study is the pronounced sensitivity of mGluR-mediated responses to cytoplasmic dialysis. This phenomenon may explain why in most studies using whole-cell patch recording, concentrations of agonists to activate postsynaptic mGluRs exceed EC_{50} values for group I mGluRs (Conn and Pin, 1997) by one to two orders of magnitude. The rundown of metabotropic responses can be avoided with the perforated patch-clamp method (Horn and Marty, 1988), but this approach is technically demanding. Here, we show that a simpler method employing patch pipettes with a higher resistance (10 to 15 MΩ) allows mGluR-mediated currents to be recorded with similar amplitudes to those obtained with the perforated patch-clamp technique.

In conclusion, we have shown that even under conditions where cytoplasmic dialysis is minimized, mGluR-LTD is not observed in CA3-CA1 pyramidal cell pairs. Temporally contiguous input from multiple Schaffer collaterals must target a CA1 pyramidal cell to induce mGluR-mediated currents and mGluR-LTD. Interestingly, the requirement for multiple inputs does not reflect a cooperative mechanism involving synaptic spillover of glutamate (Arnth-Jensen et al., 2002), but rather is consistent with a sparse expression of functional mGluRs in the dendritic tree of pyramidal cells.

Chapter 4

Group I metabotropic glutamate receptors modulate hippocampal rhythmic activity

4.1 Abstract

In this study, we check the activation conditions of group I metabotropic glutamate receptor-mediated currents in hippocampal interneurons. We show that activation of a single CA3 pyramidal cell does not evoke a somatic current mediated by group I mGluRs in a synaptically connected interneuron. Hippocampal interneurons are required for induction and propagation of theta rhythm. Our observations show that group I mGluRs in hippocampal interneurons are activated during theta oscillations and modulate the frequency of these oscillations.

4.2 Introduction

In the behaving animals, synchronous oscillations of the membrane potential of large neuronal populations can occur at various frequencies in the hippocampus. Theta rhythm (4-15 Hz) (Bland 1986; Lopes da Silva et al. 1990; Stewart and Fox 1990; Vinogradova 1995; Vertes and Kocsis 1997; Kamondi et al., 1998; Harris et al., 2001; Buzsáki, 2002) is present mainly during locomotion and other voluntary movements (Grastyán et al., 1959; Vanderwolf, 1969) and rapid eye movement (REM) sleep (Jouvet, 1969). Theta waves have been also assumed to carry mnemonic processes (Lisman and Idiart 1995; Raghavachari et al. 2001). Loss of hippocampal theta rhythm results in spatial memory deficits in the rat (Winson 1978).

Mechanisms of the generation of theta rhythm in the hippocampus are not completely understood yet. However, it is clear that the reciprocal connection between the medial septum-diagonal band of Broca (MS-DBB) and the hippocampus plays an essential role (Green and Arduni, 1954; Petsche et al., 1962; Lawson and Bland, 1993; Sik et al., 1994; Markowska et al., 1995; King et

al., 1998; Dragoi et al., 1999; Buzsáki 2002; Gulyas et al., 2003). Hippocampal interneurons represent the only output from the hippocampus to the MS-DBB (Toth et al., 1993) and are required for the induction and propagation of theta rhythm (Buzsáki et al., 1983; Sik et al., 1994; Freund and Antal, 1988; Buzsáki and Chrobak, 1995; Cobb et al., 1995; Dragoi et al., 1999; Buzsáki 2002; Gulyas et al., 2003; Mann and Paulsen, 2007). One hippocampal interneuron innervates more than 1,000 pyramidal cells (Halasy et al., 1996). Thus, they are in a key position to synchronize the activity of pyramidal cells.

Theta rhythm can be mimicked *in vitro* by activation of muscarinic acetylcholine receptors (mAchRs) (Konopacki et al., 1987). In hippocampal slice cultures, rhythmic activity can be induced with very low (10-20 nM) concentrations of the mAChR agonist methacholine (Fische et al., 1999, 2002). In this study, we show that group I mGluRs in hippocampal interneurons are activated during theta oscillations and modulate the frequency of these oscillations.

4.3 Materials and Methods

4.3.1 Slice cultures and electrophysiology

For the preparation of hippocampal organotypic slice cultures, see chapter 2.

Whole-cell voltage-clamp recordings were obtained with an Axopatch 200B amplifier (Molecular Devices). Recording pipettes were filled with (in mM) 120 K-gluconate, 10 L-glutamic acid, 5 KCl, 10 Hepes, 10 EGTA, 2 MgATP, 5 creatine phosphate (CrP), 0.4 NaGTP, 0.07 $CaCL_2$ (pH 7.2, ~290 mOsm). Membrane potentials were corrected for liquid junction potentials. Series resistance and input resistance were monitored regularly. Currents were filtered at 5 kHz and

analyzed off-line (pClamp 9, Molecular Devices). All numerical data are presented as the mean ± SEM. Significance was tested using two-tailed t-test.

4.3.2 Extracellular stimulation

For monopolar extracellular stimulation, glass pipettes filled with ACSF were placed 100-200 μm away from the recorded cell to stimulate Schaffer collaterals. To evoke a current mediated by group I mGluRs, a single-pulse stimulation (10-50 μA, 100 μs) was delivered. CA1 interneurons were clamped at -50 mV. AMPA, NMDA, $GABA_A$ and $GABA_B$ receptors were blocked by CNQX (50 μM), D-AP5 (100 μM), picrotoxin (100 μM) and CGP 62349 (5 μM), respectively.

4.3.3 Paired recordings

For current-clamp recording, pipettes (3-5 MΩ) were filled with (in mM) 120 K-gluconate, 10 L-glutamic acid, 5 KCl, 10 Hepes, 1 EGTA, 2 MgATP, 5 creatine phosphate, 0.4 NaGTP, 0.07 $CaCL_2$ (pH 7.2, ~290 mOsm). For voltage-clamp recordings, pipettes (10-15 MΩ) were filled with (in mM) 126.6 CsF, 8.4 CsCl, 10 Hepes, 10 EGTA, 2 MgATP, 5 creatine phosphate, 0.4 NaGTP (pH 7.2, ~290 mOsm).

4.3.4 Drugs

CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), D-AP5 (D-(-)-2-amino-5-phosphonopentanoic acid), (S)-MCPG ((S)-α-methyl-4-carboxyphenylglycine) and MPEP (2-methyl-6-(phenylethynyl)pyridine hydrochloride) were purchased from Ascent Scientific (Bristol, UK). DL-TBOA (DL-threo-b-benzyloxyaspartic acid) and YM 29819 was purchased from Tocris (Bristol, UK). CGP 62349 was kindly provided by Novartis (Basel, Switzerland). All other chemicals were purchased from Sigma/Fluka.

4.4 Results

4.4.1 Activation of a single CA3 pyramidal cell does not evoke a somatic current mediated by group I mGluRs in a synaptically connected interneuron

We checked whether a group I mGluR-mediated current could be activated in a hippocampal interneuron in stratum radiatum or stratum oriens of the CA3 region when only a single presynaptically connected CA3 pyramidal cell is evoked to fire. High resistance pipettes (10-15 MΩ) were employed to minimize cytoplasmic dialysis, which has been shown to be critical for induction of group I mGluR-mediated responses (see chapter 3). Following the blockade of NMDA, AMPA/kainate, $GABA_A$ and $GABA_B$ receptors, even a high-frequency train of 30 APs at 100 Hz failed to evoke a metabotropic response in an interneuron voltage-clamped at -50 mV, a potential where mGluR-mediated currents are maximal (Gee et al., 2003) (n = 3; Fig. 4.1, lower). As postsynaptic mGluRs in hippocampal interneurons are localized in perisynaptic and extrasynaptic zones (Lujan et al., 1996, 1997), membrane transporters may prevent glutamate access to mGluRs. However, in the presence of the glutamate transporter inhibitor TBOA (50 μM) (Jabaudon et al., 1999), a metabotropic response still could not be evoked (n = 3; Fig. 4.1, lower).

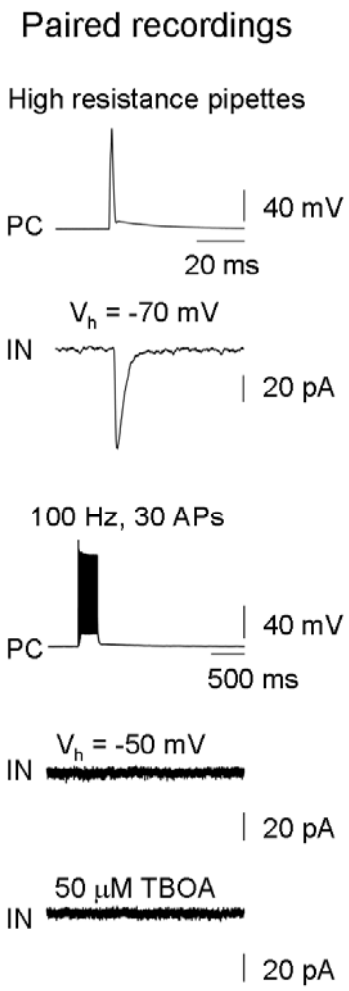

Figure 4.1 Activation of a single CA3 pyramidal cell does not evoke a current mediated by group I mGluRs in a connected interneuron of the CA3 region. Postsynaptic interneurons were voltage-clamped at -50 mV and recording pipettes with a resistance of 10-15 MΩ were used. Upper, one AP triggered in a CA3 pyramidal cell elicited a fast EPSC in a connected CA1 interneuron (V_h = -70 mV). Lower, blocking ionotropic receptors (100 μM D-AP5, 50 μM CNQX) and $GABA_A$ and $GABA_B$ receptors (100 μM picrotoxin, 5 μM CGP 62349) failed to reveal a slow metabotropic current in response to a 100 Hz train of 30 APs. Inhibition of glutamate transporters (50 μM TBOA) under these conditions also did not reveal an mGluR-mediated current. Traces represent averages of 10 sweeps.

4.4.2 Extracellular stimulation of Schaffer collaterals evokes group I mGluR-mediated EPSCs in interneurons of the CA1 region

With extracellular stimulation of Schaffer collaterals (100 Hz, 4 pulses, 20-50 μA, 100 μs duration), mGluR-mediated EPSCs were consistently evoked in interneurons of the CA1 region (n = 4; Fig. 4.2A, B). These EPSCs were blocked by the group I mGluR antagonist (S)-MCPG (500 μM, n = 3; Fig. 4.2B).

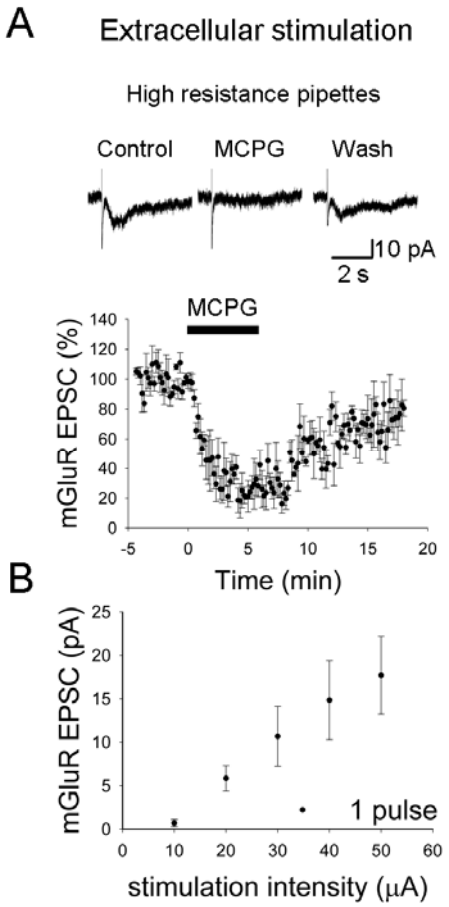

Figure 4.2 Extracellular stimulation of Schaffer collaterals evokes group I mGluR-mediated EPSCs in interneurons of the CA1 region. (A) Upper, extracellular stimulation of Schaffer collaterals (100 Hz, 4 pulses, 10-50 μA, V_h = -50 mV) evoked an mGluR-mediated current in CA1 interneurons when using high resistance recording pipettes (10-15 MΩ). Lower, the slow EPSC was significantly reduced by (S)-MCPG (500 μM, n = 3). Responses were elicited at 0.1 Hz. Traces represent averages of 5 sweeps. (B) Average amplitudes of mGluR EPSCs evoked with extracellular stimulation (100 Hz, 2 pulses, 10-50 μA, V_h = -50 mV; n = 4).

4.4.3 Group I mGluRs modulate hippocampal theta rhythm

More than 1,000 pyramidal cells terminate on a single interneuron in hippocampus (Hayashi et al., 1993). Although a single CA3 pyramidal cell does not activate group I mGluRs in a synaptically connected interneuron, concurrent activation of several pyramidal cells may activate group I mGluRs in hippocampal interneurons. During theta oscillations approximately 2% of pyramidal cells fire together (Csicsvari et al. 1998). Therefore, we hypothesize that group I mGluRs in hippocampal interneurons are activated during theta oscillations.

Rhythmic activity was induced with 50 nM concentration of the muscarinic acetylcholine receptor agonist methacholine (Fische et al., 1999, 2002). Application of mGlurR1 antagonist YM 298198 (1 μM) and mGlurR5 antagonist MPEP (10 μM) decreased the frequency of theta oscillations (for YM 298198, 61 ± 7% of baseline, n = 7, $p<0.05$; for MPEP, 51 ± 4% of baseline, n=9, $p<0.001$; Figure 4.3). The above data show that group I mGluRs in hippocampal interneurons are activated during theta oscillations.

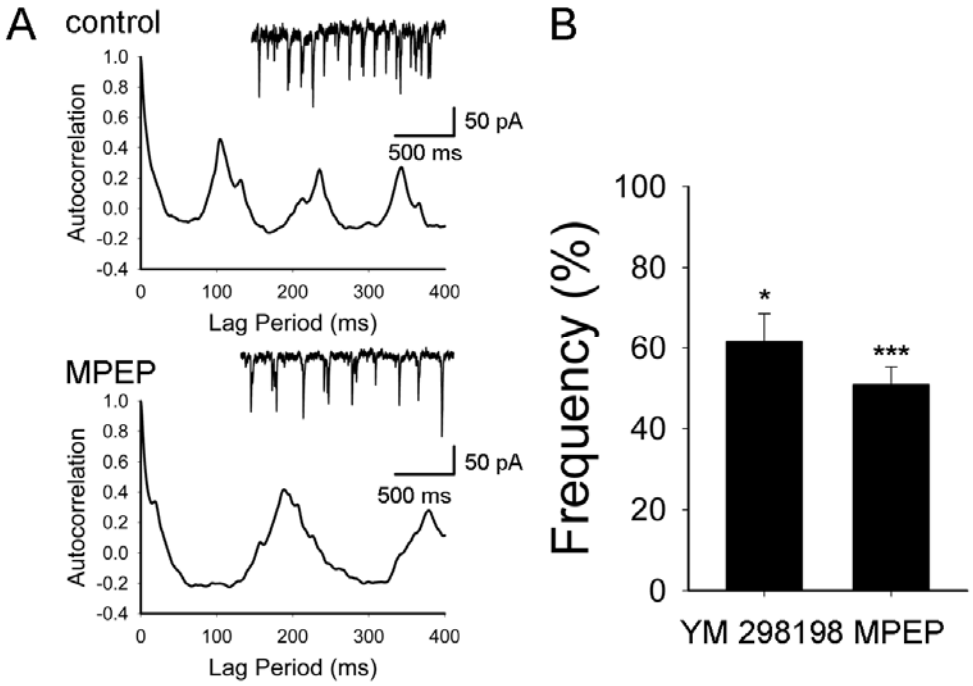

Figure 4.3 Group I metabotropic glutamate receptors modulate hippocampal rhythmic activity. (A) Application of the mGlurR5 antagonist MPEP (10 μM) decreased the frequency of oscillations as illustrated in the autocorrelograms. Inset, representative traces recorded from a CA1 pyramidal cell voltage clamped at -70 mV. (b) The frequency of the oscillations was decreased by the mGlurR1 antagonist YM 298198 (1 μM, n = 7) and by the mGlurR5 antagonist MPEP (10 μM, n = 9); *$p < 0.05$ and ***$p < 0.001$.

4.5 Discussion

In this study, we report that that activation of a single CA3 pyramidal cell does not evoke a somatic current mediated by group I mGluRs in a synaptically connected interneuron. However, mGluR-mediated EPSCs were consistently evoked with extracellular stimulation of Schaffer collaterals. This is reminiscent of the result in chapter 3, which is, concurrent activation of ~8 CA3 pyramidal cells is required to activate group I mGluRs in a CA1 pyramidal. Therefore, we checked whether concurrent activation of several pyramidal cells are necessary for activation of group I mGluRs in interneurons. We show that block of group I mGluRs modulates the frequency of theta oscillations, which proves that group I mGluRs in interneurons are activated by synchronized activity of pyramidal cells during hippocampal theta rhythm.

Although mGluR1 and mGluR5 are highly homologous, their function (Valenti et al., 2002) and distribution (see chapter 1.3) are reported to be different. Our data show that blockade of mGluR1 or mGluR5 can reduce the frequency of hippocampal theta oscillations.

Hippocampal interneurons are required for the induction and propagation of theta rhythm (Buzsáki et al., 1983; Sik et al., 1994; Freund and Antal, 1988; Buzsáki and Chrobak, 1995; Cobb et al., 1995; Dragoi et al., 1999; Buzsáki 2002; Gulyas et al., 2003; Mann and Paulsen, 2007). However, rhythmic activity relies on the balance between inhibition and excitation (Shu et al., 2003; Mann and Paulsen, 2007). It would be interesting to compare the functions of group I mGluRs located in pyramidal cells and in interneurons. Up to now, there is no pharmacological tool that can selectively block or activate group I mGluRs selectively only in pyramidal cells or only in interneurons. Transgenic mice in which the mGluR1 or/and mGluR5 coding gene is knocked out selectively from pyramidal cells or from interneurons would be useful tools to study this question.

Chapter 5

Conclusion and perspectives

5.1 Conclusion and discussion

My thesis project was to determine the conditions required for activation of group I mGluRs in rat hippocampus.

Firstly, as described in chapter 2, I checked in hippocampal slice cultures whether the ambient glutamate concentration in the extracellular space (typically 2 μM in the hippocampal slices) is sufficient to tonically activate group I mGluRs (whose EC_{50} is ~10 μM). However, I show that group I mGluRs in CA1 pyramidal cells are not tonically activated by ambient glutamate in hippocampal slice cultures. One possible explanation is that the background level of extracellular glutamate in hippocampal organotypic slice cultures is lower than in the brain *in vivo* or in acute brain slices. Another possible explanation is that the ambient concentration of glutamate in acute hippocampal slices is much lower than the value formerly reported. In a new measurement, the ambient concentration of glutamate in acute hippocampal slices was reported to be ~25 nM (Herman and Jahr, 2007).

In chapter 3, I present my investigation on the electrical stimulation conditions to activate group I mGluRs in CA1 pyramidal cells. NMDAR-LTD and mGluR-LTD have both been found in CA1 pyramidal cells. For the induction of NMDAR-LTD, cooperative interactions of multiple presynaptic inputs are not essential, such that appropriately timed discharge of a single CA3 pyramidal cell can lead to homosynaptic LTD in a targeted CA1 pyramidal cell (Debanne et al., 1996). Activation of group I mGluRs with low frequency stimulation of many Schaffer collaterals can induce mGluR-LTD in CA1 pyramidal cells. In chapter 3, I show that activation of a single CA3 pyramidal cell does not induce mGluR-LTD in a connected CA1 pyramidal cell. An estimate based on data obtained from paired recording and extracellular stimulation indicates that 8 active CA3 inputs are required to induce mGluR-LTD in a targeted CA1 pyramidal cell. Similarly, I show that activation of a single CA3 pyramidal cell does not evoke a somatic current

mediated by mGluRs in a connected CA1 pyramidal cell. An estimate based on data obtained from paired recording and extracellular stimulation indicates that 20 CA3 pyramidal cell axons targeting a given CA1 pyramidal cell must be activated concurrently to induce a somatic current mediated by group I mGluRs. This may due to the sparse distribution of functional dendritic group I mGluRs, which was shown in a recent study by Holbro et al. (2009). The authors showed that responses mediated by group I mGluR in CA1 pyramidal cells could only be induced in a subset of endoplasmic reticulum-containing mushroom spines, which represent less than 10% of all spines. Furthermore, the failure rate with glutamate uncaging for mGluR-mediated responses in these mGluR-positive spines was approximately 80%.

I show that 8 and 20 CA3 inputs are required to induce mGluR-LTD and an mGluR-mediated current in a targeted CA1 pyramidal cell, respectively. This is, actually, a relatively small number considering that a CA1 pyramidal cell *in vivo* receives, on average, input from 5,500 CA3 pyramidal cells (Amaral et al., 1990).

In my investigation of the conditions to activate mGluR-mediated responses in CA1 pyramidal cells, an interesting finding was the pronounced sensitivity of mGluR-mediated responses to cytoplasmic dialysis associated with conventional whole-cell patch clamp recording. The rundown of metabotropic responses can be avoided by using the perforated patch-clamp method (Horn and Marty, 1988), but this approach is technically very demanding. Here, I show that a simpler method, in which patch pipettes with a resistance of 10 to 15 MΩ instead of 3 to 5 MΩ are employed, allow mGluR-mediated currents to be recorded with similar amplitudes to those obtained with the perforated patch-clamp technique.

Finally, in chapter 4, I check the activation conditions of group I metabotropic glutamate receptor-mediated currents in hippocampal interneurons. I show that although extracellular stimulation of Schaffer collaterals evokes group I mGluR-mediated EPSCs in interneurons of the CA1 region, activation of a single CA3

pyramidal cell does not evoke a somatic current mediated by group I mGluRs in a synaptically connected interneuron. This is reminiscent of the observations in chapter 3, in which I found that concurrent activation of CA3 pyramidal cells is required to activate group I mGluRs in a CA1 pyramidal cell. I find that block of group I mGluRs modulates the frequency of hippocampal theta oscillations, which proves that group I mGluRs in interneurons are activated by synchronized activity of pyramidal cells during hippocampal theta rhythm. This finding is consistent with the fact that more than 1,000 pyramidal cells terminate on a single interneuron in hippocampus (Hayashi et al., 1993) and during theta oscillations approximately 2% of pyramidal cells fire together (Csicsvari et al. 1998).

5.2 Perspectives

In the study of Holbro et al., 2009, taking advantage of a two-photon glutamate uncaging method, the authors can identify mGluR-positive spines and then specifically stimulate an identified spine to obtain mGluR-LTD. However, it has not been possible to identify mGluR-positive synapses with paired recordings. Furthermore, localized glutamate uncaging may be more efficient than synaptically released glutamate at reaching peripherally localized mGluRs. In chapter 3, I show that less CA3 inputs (8 v.s. 20) are required to induce mGluR-LTD than a somatic mGluR-mediated current in a targeted CA1 pyramidal cell. An mGluR-dependent calcium signal to initiate LTD induction may have a lower stimulation threshold than an mGluR-mediated current, as has been demonstrated in cerebellar Purkinje cells (Finch and Augustine, 1998; Takechi et al., 1998). At present, it is not possible to repeat this type of experiment in CA3-CA1 paired recordings as it is difficult to localize the very few activated spines in the large dendritic tree of a CA1 pyramidal cell. Two-photon glutamate uncaging can be a practical method to address this question.

Hippocampal interneurons are required for the induction and propagation of theta rhythm (Buzsáki et al., 1983; Sik et al., 1994; Freund and Antal, 1988; Buzsáki and Chrobak, 1995; Cobb et al., 1995; Dragoi et al., 1999; Buzsáki 2002; Gulyas et al., 2003; Mann and Paulsen, 2007). However, rhythmic activity relies on the balance between inhibition and excitation (Shu et al., 2003; Mann and Paulsen, 2007). It would be interesting to compare the functions of group I mGluRs located in pyramidal cells and in interneurons. Up to now, there is no pharmacological tool that can selectively block or activate group I mGluRs selectively only in pyramidal cells or only in interneurons. Transgenic mice in which the mGluR1 or/and mGluR5 coding gene is knocked out selectively from pyramidal cells or from interneurons would be useful tools to study this question.

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List of abbreviations

ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPAR	AMPA receptor
ATP	adenosine triphosphate
CaM	calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
CF	climbing fiber
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
D-AP5	D-(-)-2-amino-5-phosphonopentanoic acid
DHPG	dihydroxyphenylglycine
EPSC	excitotory postsynaptic current
GABA	γ-aminobutyric acid
GPCR	G-protein-coupled receptor
GRIP	glutamate receptor interacting protein
GTP	guanosine triphosphate
iGluRs	ionotropic glutamate receptors
IN	interneuron
ING	interneuron network gamma
IP_3	inositol-1,4,5-trisphosphate
KAR	kainate receptor
L-AP4	L-(+)-2-amino-4-phosphonobutyrate
LFS	low frequency stimulation
LTD	long-term depression
LTP	long-term potentiation
mAChR	muscarinic acetylcholine receptor
MCPG	α-methyl-4-carboxyphenylglycine
mGluR	metabotropic glutamate receptor
MPEP	2-methyl-6-(phenylethynyl)pyridine hydrochloride

MS-DBB	medial septum-diagonal band of Broca
NBQX	2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide)
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
PC	pyramidal cell
PF	parallel fiber
PICK1	protein interacting with C kinase 1
PKC	protein kinase C
PLC	phospholipase C
PN	Purkinje neuron
PSD	postsynaptic density
REM	rapid eye movement
SEM	standard error of mean
Siah-1A	seven in absentia homolog-1A
TBOA	threo-b-benzyloxyaspartic acid
TTX	tetrodotoxin
VFO	very fast oscillation

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